Identification of early biomarkers of metabolic functions with implications on health and related to obesity using animal models of intervention in early life

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INDEX

Abbreviations

αMSH , alpha-melanocyte-stimulating hormone
ACC1, acetyl-coenzyme A carboxylase alpha
ACOX1, acyl-coenzyme A oxidase 1
AgRP, agouti-related peptide
ARC, arcuate nucleus
ATGL, adipose triglyceride lipase
ATP, adenosine triphosphate
BAT, brown adipose tissue
cAMP, cyclic adenosine monophosphate
CART, cocaine- and amphetamine-regulated transcript
CNS, central nervous system
CPT1A, carnitine palmitoyltransferase 1alpha
CPT1B, and carnitine palmitoyltransferase 1 beta
CRH, corticotrophin releasing hormone
CRMP1, collapsin response mediator protein 1
DIO2, deiodinase iodothyronine type II
FA, fatty acids
FASN, fatty acid synthase
GI, gastrointestinal tract
GLA, galactosidase, alpha
GLS, glutaminase
HF, high-fat
HOMA, the homeostasis model assessment
HSL/LIPE, hormone-sensitive lipase
IGF-1, insulin-like growth factor 1
INSR, insulin receptor
IRS1, insulin receptor substrate 1
LHA, lateral hypothalamic areas
LPL, lipoprotein lipase
LRP1, low density lipoprotein receptor-related protein 1
LRP11, low density lipoprotein receptor-related protein 11
MCH, melanin concentrating hormone
mRNA, messenger RNA
NE, norepinephrine
NF, normal-fat
NPY, neuropeptide Y
OBRB, long-form leptin receptor
OXA, orexin-A
PAOX, polyamine oxidase (exo-N4-amino)
PBMCs, peripheral blood mononucleated cells
PFA, perifornical area
PGC1A, peroxisome proliferator activated receptor gamma coactivator 1 alpha
PKA, protein kinase A
POMC, pro-opiomelanocortin

PPARα, peroxisome proliferator activated receptor alpha
PPARγ, peroxisome proliferator activated receptor gamma
PVN, paraventicular nucleus
qPCR, real-time polymerase chain reaction
RXRb, retinoid X receptor beta
SNS, sympathetic nervous system
SOCS-3, suppressor of cytokine signalling-3
SREBP1c, sterol response element binding protein 1c
STAR, steroidogenic acute regulatory protein
STAT3, signal transducer and activator of transcription 3
T3, triiodothyronine
T4, thyroxine
TG, triglycerides
TMSB4X, thymosin beta 4, X-linked
TRH, thyrotropin releasing hormone
TyrOH, tyrosine hydroxylase
UBASH3B, ubiquitin associated and SH3 domain containing, B
UCP1, uncoupling protein 1
VLDL, very low density lipoprotein
VMH, ventromedial hypothalamic nucleus
WAT, white adipose tissue
WHO, World Health Organization

Identification of early biomarkers of metabolic functions with implications on health and related to obesity using animal models of intervention in early life

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Abstract

The main aim of the present PhD thesis was to identify early transcriptome-based biomarkers of metabolic functions with implications on health (metabolic health) and those related to body weight control and obesity in peripheral blood mononuclear cells (PBMCs) of rats, which – as a result of interventions in the perinatal period – are more susceptible to obesity (model of maternal calorie restriction during pregnancy) or have developed certain protection against overweight and other associated alterations (model of maternal calorie restriction during lactation).

Results show that moderate maternal calorie restriction of 20% during the first part of gestation programs the offspring for a lower capacity to respond to insulin and to central leptin action, which leads to hyperphagia and metabolic alterations in both genders and higher body weight in males. Moreover, these animals showed reduced brown adipose tissue burning capacity caused by both impairment of sympathetic drive and alteration in thyroid hormone signaling. It may cause the greater sensitivity to cold that undergo these animals and contribute to the higher propensity for fat accumulation and other metabolic alterations later in life. Adverse outcomes of moderate maternal calorie restriction during pregnancy could be associated with the absence of a transient rise in plasma leptin levels in offspring during the suckling period.

Oral supplementation with physiological doses of leptin during the suckling period to the offspring of calorie-restricted dams during pregnancy is able to revert, at least partly, most of the malprogrammed effects studied caused by maternal undernutrition during gestation. Specifically, leptin treatment normalized altered cellularity in the hypothalamus, and particularly the number of orexigenic NPY cells in arcuate nucleus, associated with normalization of gene expression patterns in this tissue. Moreover, leptin-treated male animals displayed restored sympathetic drive into white adipose tissue (WAT), accompanied by amelioration of catabolic capacity in this tissue. Microarray analysis performed in PBMCs

from male pups showed that, out of the 224 genes whose expression was altered due to maternal undernutrition during gestation, leptin treatment normalized the expression of 218 of them to control levels. These markers may be useful for early identification and subsequent monitoring of individuals who are at risk of later diseases and would specifically benefit with the intake of appropriate amounts of leptin during lactation.

Unlike the effects of maternal calorie restriction during gestation, moderate maternal calorie restriction of 20% during lactation in rats programs the offspring for better metabolic health in terms of body weight and lipid handling. This condition during lactation affected lipogenic and oxidative capacity in WAT and liver and increased their sensitivity to the peripheral effects of leptin and insulin. These adaptations were partially maintained throughout life but were particularly evident when animals were exposed to high-fat diet feeding. Microarray analysis of PBMCs from weaned rat identified a number of 278 genes, which expression was different in the offspring of dams submitted to calorie restriction during lactation compared to controls. Among lipid metabolism-related genes, Cpt1a, Fasn and Star emerged as particularly interesting, as their expression levels in PBMCs were significantly correlated with expression profiles in WAT and/or liver.

All in all, results from this PhD thesis enabled to identify a number of easily accessible predictive biomarkers of metabolic health and dysfunction, which may potentially provide a valid biological readout for the study of metabolic processes in humans.

Identificación de biomarcadores tempranos de funciones metabólicas con las implicaciones sobre la salud y relacionados con la obesidad en modelos animales de la intervención en la vida temprana

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Resumen

El principal objetivo de esta tesis doctoral ha sido identificar biomarcadores transcriptómicos tempranos de funciones metabólicas con implicaciones en la salud (salud metabólica) y aquellos relacionados con el control del peso corporal y la obesidad en células mononucleares de sangre periférica (PBMCs) de ratas, las cuales - como resultado de intervenciones en el periodo perinatal - son más susceptibles de padecer obesidad (modelo de restricción calórica materna durante el embarazo) o han desarrollado una cierta protección frente al sobrepeso y otras alteraciones asociadas (modelo de restricción calórica materna durante la lactancia).

Los resultados muestran que una restricción calórica materna moderada del 20% durante la primera parte de la gestación programa a la descendencia para una menor capacidad de respuesta a la insulina y a la acción central de la leptina, lo cual desencadena hiperfagia y alteraciones metabólicas en ambos sexos y un mayor peso corporal en los machos. Además, estos animales mostraron una menor capacidad termogénica en el tejido adiposo marrón causada por una deficiencia en la inervación simpática y una alteración en la señalización de la hormona tiroidea. Esto podría causar la mayor sensibilidad al frio que sufren estos animales y contribuir a la mayor propensión a la acumulación de grasa y otras alteraciones metabólicas a lo largo de la vida. Los efectos adversos de una restricción calórica moderada durante la gestación podrían estar asociados a la ausencia de un aumento transitorio en la concentración plasmática de leptina en las crías durante el período de lactancia.

La suplementación oral con dosis fisiológicas de leptina durante el periodo de lactancia a las crías de madres sometidas a restricción calórica durante el embarazo es capaz de revertir, al menos en parte, la mayoría de los efectos adversos estudiados causados por una nutrición insuficiente durante la gestación. En concreto, el tratamiento con leptina normalizó la alterada celularidad en el hipotálamo, y en particular el número de células orexigénicas NPY en el núcleo arqueado, lo cual se asoció a la normalización de los patrones de expresión génica en

este tejido. Además, los animales machos tratados con leptina mostraron una restauración de la señalización simpática en tejido adiposo blanco (TAB), acompañada por una mejora de la capacidad catabólica en este tejido. El análisis de microarray realizado en PBMCs de crías macho mostró que, de los 224 genes cuya expresión se vio alterada debido a la malnutrición materna durante la gestación, el tratamiento con leptina durante la lactancia normalizó la expresión de 218 a los niveles controles. Estos marcadores podrían ser útiles para la identificación temprana y el posterior seguimiento de los individuos que están en riesgo de padecer futuras enfermedades y que podrían beneficiarse especialmente con la ingesta de cantidades apropiadas de leptina durante la lactancia.

A diferencia de los efectos de una restricción calórica materna durante la gestación, una restricción calórica materna moderada del 20% durante la lactancia en ratas programa a la descendencia para una mejor salud metabólica en términos de peso corporal y de procesamiento de los lípidos. Esta condición durante la lactancia afectó la capacidad lipogénica y oxidativa en el TAB e hígado e incrementó su sensibilidad a los efectos periféricos de la leptina y la insulina. Dichas adaptaciones se mantuvieron parcialmente a lo largo de la vida pero fueron particularmente evidentes cuando los animales se expusieron a una dieta rica en grasa. El análisis de microarray realizado en PBMCs de las ratas en el destete permitió identificar 278 genes con diferente expresión en las crías de las madres sometidas a restricción calórica durante la lactancia respecto de las controles. Entre los genes relacionados con el metabolismo lipídico, Cptla, Fasn y Star destacaron como particularmente interesantes, ya que su expresión en PBMCs se correlacionó significativamente con la expresión en TAB y/o hígado.

En conclusión, los resultados de esta tesis doctoral han permitido identificar una serie de biomarcadores fácilmente accesibles y predictibles de salud o disfunción metabólica, pudiendo proporcionar una potencial herramienta biológica válida para el estudio de procesos metabólicos en humanos.

List of original articles

This thesis is based on 7 original research manuscripts (numbers 1-7, listed below) and 1 patent (number 8, see proof of submission in Annex I).

1. Palou, M., Konieczna, J., Torrens, J.M., Sánchez, J., Priego, T., Fernandes, M.L., Palou, A., and Picó, C. **Impaired insulin and leptin sensitivity in the offspring of moderate caloric-restricted dams during gestation is early programmed.** *J Nutr Biochem,* 2012;23(12):1627-1639

2. Palou, M., Priego, T., Romero, M., Szostaczuk, N., Konieczna, J., Cabrer, C., Remesar, X., Palou, A., and Picó, C. **Moderate calorie restriction during gestation programs offspring for lower BAT thermogenic capacity driven by thyroid and sympathetic signalling.** *Int J Obes (Lond),* 2014; doi: 10.1038/ijo.2014.56. Epub ahead of print

3. Konieczna, J., Garcia, A.P., Sánchez, J., Palou, M., Palou, A., and Picó, C. **Oral leptin treatment in suckling rats ameliorates detrimental effects in hypothalamic structure and function caused by maternal caloric restriction during gestation.** *PLoS One,* 2013;8(11):e81906

4. Konieczna, J., Palou, M., Sánchez, J., Picó, C., and Palou, A. **Leptin supplementation in suckling rats recuperates altered white adipose tissue sympathetic innervation and function caused by maternal caloric restriction during gestation.** Manuscript to be submitted for publication

5. Konieczna, J., Sánchez, J., Palou, M., Picó, C., and Palou, A. **Leptin supplementation in suckling rats reverts blood cell transcriptomic-based potential early biomarkers of adverse programming effects associated to gestational calorie restriction.** Manuscript to be submitted for publication

6. Torrens, J.M., Konieczna, J., Palou, M., Sánchez, J., Picó, C., and Palou, A. **Early biomarkers identified in a rat model of a healthier phenotype based on early postnatal dietary intervention may predict the response to an obesogenic environment in adulthood.** *J Nutr Biochem,* 2014;25(2):208–218

7. Konieczna, J., Sánchez, J., van Schothorst, E.M., Torrens, J.M., Bunschoten, A., Palou, M., Picó, C., Keijer, J., and Palou, A. **Identification of early transcriptome-based biomarkers related to lipid metabolism in peripheral blood mononuclear**

cells of rats nutritionally programmed for improved metabolic health. *Genes Nutr* 2014;9(1): 366

8. Palou, A., Picó, C., Konieczna, J., Sánchez, J., and Palou, M. **Método para la predicción y/o la prevención de sobrepeso, obesidad y/o sus complicaciones mediante análisis de expresión génica/ Method for prediction and/or prevention of overweight, obesity and/or its complications through gene expression analysis.** Patent (Request reference: P201430428)

I. INTRODUCTION

INTRODUCTION

Nowadays, obesity is life-threatening worldwide phenomenon. It is estimated that approximately 1000 million adults are overweight and at least 475 million adults are obese worldwide (IOTF 2010). In European Union countries, overweight affects 30-70% of the adult population, while obesity affects 10-30% of adults (WHO 2008). Obesity decreases the quality of life and implies high health costs as a consequence of its co-morbidities. Overweight and obesity constitute risk factors for a number of chronic diseases including diabetes, cardiovascular diseases (heart disease and stroke), musculoskeletal disorders (osteoarthritis), some cancers, which often lead to premature death (WHO 2008). Therefore, elucidation of the potential causes, as well as determination of disease treatment, and probably even more its prevention is markedly important.

It is widely agreed, that obesity results from a prolonged imbalance between energy intake and energy expenditure. Mostly, the reason of this metabolic pathology is attributed to the interaction between unhealthy lifestyle factors, such as a lack of physical activity and excessive calorie intake, and genetic factors (Chan and Woo 2010). Despite mentioned key components in the development of obesity, there is increasing number of studies which indicate that early life nutritional environment may play an important role programming the risk of obesity and other features of the metabolic syndrome in adult life (McMillen and Robinson 2005; Pico et al. 2012). Thus, pregnancy and lactation are revealed as critical periods, where nutritional manipulations may lead to permanent adaptations with lasting effects on metabolic mechanisms in the offspring, influencing the propensity to obesity and related metabolic alterations in adult life.

Knowing the risk of obesity and related metabolic alterations appears to be essential to implement effective preventive strategies. In this regard, identification of early biomarkers may provide a diagnostic tool to detect and monitor emerging perturbations related to obesity already at early stages of life. Moreover, it is known that obesity-related risk can be prevented by changes in diet. Thus, development of novel biomarkers of the effects of food based-strategies on metabolic or phenotypic features, may serve as biological indicators of both decreased/increased risk of body weight-related disorders and of response to therapy used.

The individual sections of present introduction address some of the aspects related to obesity that might help to develop nutrigenomic approaches to prevent this current epidemic. In the

section 1, main issues related to maintenance of energy homeostasis are described. In the section 2, nutritional conditions during early life, gestation and lactation, which are known to affect the likelihood of developing obesity and other metabolic alterations, are reviewed. The issue related to the development of biomarkers of improved or impaired metabolic health and the use of peripheral blood cells as a source of biomarkers is addressed in the section 3.

1. Energy homeostasis

Energy homeostasis refers to all processes that aim to maintain stability of the metabolic state. It requires the integration of activities in many different body systems, including central nervous system (CNS), and particularly the hypothalamus, and a set of peripheral organs (i.e. the gastrointestinal tract, adipose tissue, skeletal muscle, liver, pancreas, etc.) that digest, convert, ship, and store nutrients and energy (Sanchez-Lasheras et al. 2010). In this section, main tissues, key genes and hormones involved in energy homeostasis are described.

1.1. Main tissues involved in maintenance of energy homeostasis

1.1.1. Hypothalamus

The hypothalamus is a region of the brain that serves as a central homeostatic regulator of numerous physiological and behavioral functions (Lee and Blackshaw 2012). Among them, the hypothalamus plays a particular role in energy homeostasis integrating and coordinating several types of signals, including hormones and nutrients, to coordinate energy intake and expenditure (Woods et al. 2008).

The hypothalamus consists of several nuclei: arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamic areas (LHA) and perifornical area (PFA) (Valassi et al. 2008). Within the ARC there are anorexigenic neurons that coexpresses alpha-melanocyte-stimulating hormone (αMSH; derived from the pro-opiomelanocortin (POMC) precursor) and cocaine- and amphetamine-regulated transcript (CART). The others are orexigenic and coexpresses neuropeptide Y (NPY) and agouti-related peptide (AgRP) (Bouret and Simerly 2006). The other brain areas located downstream the ARC are also involved in the control of food intake. PVN produces anorexigenic peptides such as thyrotropin releasing hormone (TRH), corticotrophin releasing hormone (CRH) and oxytocin; LHA and PFA secrete the orexigenic substances orexin-A (OXA) and melanin concentrating hormone (MCH) (Valassi et al. 2008).

The hypothalamus integrates peripheral and central signals to regulate metabolic status via the modulation of expression of appetite-regulating peptides (Schwartz et al. 2000). Peripheral regulation (short-term regulation of food intake) includes mechano- and chemoreceptors signaling caused by the presence and energy density of food in the gastrointestinal (GI) tract. Nutrients (e.g., amino acids and fatty acids) and peptide hormones originated from GI tract during a meal (cholecystokinin, glucagon-like peptide-1, peptide YY, gastric leptin, etc.) are also involved in short-term regulation of food intake. They reach the nucleus tractus solitarius (NTS) in the caudal brainstem, from which afferents fibers project to the ARC. However, the energy density of food and hormonal signals by themselves are insufficient to produce sustained changes in energy balance and body adiposity. Thus, in ARC, these signals interact with central regulators (long-term regulation) of food intake, namely adiposity signals (insulin and leptin), and the orexigenic gastric peptide, ghrelin, to maintain energy homeostasis. In response, neurons in the ARC send out signals in the form of neuropeptides that instruct other control centers to either stimulate or suppress the appetite (Valassi et al. 2008; Havel 2001).

1.1.2. White adipose tissue (WAT)

The excess of WAT is a widely accepted hallmark of the obese state. Hyperplasia (cell number increase) and hypertrophy (cell size increase) are two possible mechanisms that contribute to growth of adipose tissue in obesity. Regarding growing concern with obesity, adipocytes have become perceived as undesirable cells in the organism. Nevertheless, the adipose tissue, which operates as a structured whole, is a highly active organ that plays several key roles in the organism to maintain homeostasis. Thus, adipose tissue possesses the ability to modulate its own metabolic activities, including differentiation of new adipocytes and production of blood vessels as necessary to accommodate increasing fat stores (Frayn et al. 2003).

Conventionally, the most fundamental role of WAT is lipid storage, thus, lipogenesis and lipolysis raise as the main metabolic processes attributed to this organ. Adipocytes store lipids in periods of positive energy balance, which is being mobilized during food deprivation with the release of fatty acids. In this way, adipose tissue provides fuel for various organs, depending on the body's nutritional state (Frayn et al. 2003; Trayhurn and Beattie 2001). Lipids are stored in adipocytes in the form of triglycerides (TG). The capacity of WAT for *de novo* lipogenesis is relatively low. Thus, TG are being deposited in adipose tissue through the

enzymatic activity of lipoprotein lipase (LPL), responsible for hydrolysis of TG from circulating lipoproteins and uptake of fatty acids into cells for esterification. These processes are mediated by insulin postprandial action (Frayn et al. 2003). In turn, in the process of TG mobilization, sensitive to suppression by insulin, hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) play the most pivotal role. It is well established, that lipolysis in WAT is stimulated by sympathetic nervous system (SNS) that innervates this fat depot. Increased release of norepinephrine (NE) from sympathetic nerves activates β3-adrenoreceptors, stimulating adenylate cyclase. Increase in intracellular levels of cyclic adenosine monophosphate (cAMP) activates protein kinase A (PKA), which phosphorylates and activates HSL (Holm 2003). In turn ATGL, which work in conjunction with HSL, is the initiator lipase for NE-stimulated lipolysis and may be rate limiting in the mobilization of cellular fat depots. Unlike that of HSL, its activity is not dependent on PKA phosphorylation (Bartness et al. 2010).

WAT also plays an essential role in the organism due to its endocrine function. The hormone leptin was the first discovered peptide secreted by the adipose tissue. Other proteins are also secreted by WAT, such as angiotensinogen, adipsin, acylation-stimulating protein, adiponectin, retinol-binding protein, tumour neorosis factor a, interleukin 6, plasminogen activator inhibitor-1 and tissue factor, fasting-induced adipose factor, a fibrinogen-angiopoietin-related protein, metallothionein, resistin, etc. These proteins have many functions in the body, e.g. play role in inflammation process, vascular haemostasis, and obesity development (Trayhurn and Beattie 2001). In this regard, resistin has been linked with insulin resistance induction (Steppan et al. 2001), and adiponectin with insulin sensitivity enhancement (Lihn et al. 2005).

1.1.3. Brown adipose tissue (BAT)

Besides WAT, there are small amounts of BAT in the body, which is the main site of adaptive thermogenesis, producing heat in hibernating animals, newborns and cold-exposed mammals. The recent recognition of BAT presence and function in adult humans has reopened the interest for this tissue as a target for obesity therapy (Tam et al. 2012). Unlike the white adipocytes, the brown ones, are characterized by multilocular lipid droplets and a greater amount of mitochondria that express uncoupling protein 1 (UCP1) (Cinti 2005; Sell et al. 2004). UCP1 uncouples the rates of substrate oxidation and ATP production by favoring a loss of protons and energy release in the form of heat (Palou et al. 1998).

The process of thermogenesis mediated by UCP1 activation is under the control of SNS, which innervates BAT. The terminals of sympathetic nerves in BAT release NE that turn on a cascade of intracellular events. This leads to stimulation of beta adrenoreceptors ending in activation of UCP1, together with TG mobilization, thus providing the fatty acids necessary for UCP1 activation (Palou et al. 1998; Cannon and Nedergaard 2004). The expression of UCP1 is regulated by nuclear receptors and cofactors, such as peroxisome proliferator-activated receptor gamma (PPARγ) and its coactivator, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1A) (Lowell and Spiegelman 2000), as well as by metabolic hormones such as leptin and glucocorticoids (Sell et al. 2004). In addition, thyroid hormones interact with the SNS, being critical for the full activation of NE signaling cascade and of UCP1 in BAT (Silva 2006). The sympathetic activity in BAT is regulated by physiological conditions. In this sense, the exposure to cold and food intake activate SNS leading to increased UCP1 content, while fasting exerts opposite effects (Cannon and Nedergaard 2004). Therefore, activation of BAT thermogenesis represents a mechanism that enables dissipating, in a regulated manner, part of the energy from food as heat instead of accumulating it as fat.

1.1.4. Liver

The liver is the multifunctional organ playing essential role in metabolism, bile acid production, detoxification, immunological processes, storage of energy and many essential nutrients, such as vitamins and minerals obtained from blood, production of serum proteins and hormones, and many other functions. Liver is formed by two main cell types, namely Kupffer cells that are a type of macrophage, and hepatocytes that play a major role in a variety of metabolic processes. All of the blood leaving the digestive system passes through the hepatic portal vein, making the liver responsible for metabolizing carbohydrate, lipids, and proteins into biologically useful materials.

Hepatocytes are responsible for glucose synthesis in the fasting state, and glucose uptake, storage, and utilization in the fed state. In the normal individual, the intake of a mixed meal results in modest hyperglycemia, accompanied by substantial storage of glycogen in the liver. The postprandial period is characterized by changes in hormone secretion and neural signals that combine to direct the partitioning of the glucose load among various tissues (Moore et al. 2012). The fasted-to-fed transition or vice versa, normally initiated by insulin or glucagon, respectively, includes modulation of multiple metabolic pathways in the liver in a

coordinated manner. Genes, which expression is normally induced in the fed state, include glucokinase (Gk), liver pyruvate kinase (Pklr), 6-phosphofructo-2-kinase/fructose-2,6-bisphos phatase (Pfk2), acetyl-coenzyme A carboxylase, and fatty acid synthase (Fasn). Conversely, genes that are activated during the fasted state, such as phosphoenolpyruvate carboxykinase (Pepck), fructose-1,6-bisphosphatase, and carnitine palmitoyltransferase (Cpt) 1 and 2, are downregulated in the fed state (Collier and Scott 2004).

In the fasting state, the fuel sources of the body shift from carbohydrates and fats to mostly fats. Fatty acids that were stored during feeding are released from the adipocytes and taken up by liver. There they are either reesterified to TGs and assembled into very low density lipoprotein (VLDL) or broken down through β-oxidation and used to generate ketone bodies (Lee et al. 2003). Peroxisome proliferator-activated receptor alpha (PPARα) directly regulates genes involved in hepatic fatty acid uptake – fatty acid transport protein (Fatp), fatty acid binding protein (Fabp); plasma VLDL triglyceride hydrolysis – lipoprotein lipase (Lpl); and β–oxidation – Cpt1a, and acyl-CoA oxidase 1 (Acox1) (Lee et al. 2003; McIntosh et al. 2013; Martin et al. 2009) .

1.2. Key genes involved in lipid metabolism in liver and WAT

Liver and WAT play a major role in lipid metabolism. Lipids may be synthesized (lipogenesis), mobilized (lipolysis) or oxidized (β-oxidation) in response to different stimuli such as nutritional environment (diet, and its specific components), circulating hormones, and physiological state (such as fed/fasting). The regulation of metabolic pathways involves the rapid modulation of the activity of specific proteins (enzymes, transporters), but also, on a longer-term basis, changes in their quantities. This can be achieved by modulating their transcription rate or post-transcriptional steps such as mRNA half-life and translation efficiency (Foufelle and Ferre 2002). The transcription factors belonging to the peroxisome proliferator-activated receptors (PPARs) and sterol regulatory element binding proteins (SREBPs) families are the key elements in the lipid metabolism processes that control expression of their target genes.

1.2.1. Lipogenesis

The family of membrane-bound transcription factors, SREBs, which include SREBP1a, SREBP1c, and SREBP2, has been found to regulate gene expression of certain enzymes implicated in cholesterol, lipid, and glucose metabolism. SREBP1 are mostly involved in

activation of genes related to fatty acid metabolism and *de novo* lipogenesis, whereas SREBP2 may be more selective for genes directly involved in cholesterol homeostasis. Among SREBP1, SREBP1c is expressed in most of the tissues of mice and humans, with especially high levels in the liver and WAT. Although SREBP1c expression is known to be controlled by the nutritional environment, several lines of evidence suggest that its transcription, maturation and activity is regulated by insulin (Foufelle and Ferre 2002; Osborne 2000). It is considered as the major factor involved in the nutritional regulation of lipogenesis, being involved in the control of lipogenic genes such as those coding for FAS, acetyl coenzyme-A carboxylase (ACC) (Gosmain et al. 2005), stearoyl-CoA desaturase 1 (SCD1) (Hoashi et al. 2007), as well as hexokinase-2 (HKII) involved in glucose metabolism (Gosmain et al. 2005).

The three types of PPAR receptors, termed alpha, delta (or beta), and gamma, belong to the nuclear receptor superfamily, which play a significant role in fatty acids metabolism (Schoonjans et al. 1996). PPARγ is the isoform involved in adipose differentiation process (by activation of PPARγ2), the pathogenesis of obesity and non-insulin-dependent diabetes mellitus. The main role of PPARγ in lipid metabolism is related to lipogenesis activation, as it stimulates cellular fatty acid uptake by activation of LPL, and conversion to acyl-CoA derivatives due to increased expression of genes for fatty acid transport protein and acyl-CoA synthetase (Schoonjans et al. 1996). LPL, a member of the lipase gene family, is widely distributed in many of peripheral tissues, including adipose tissue (white/brown) and muscle. LPL actions at the luminal surface o capillary endothelial cells where the enzyme hydrolyses triglycerides in circulating lipoprotein particles, chylomicrons (from intestine) and VLDL (from liver), into free fatty acids that are supplied into WAT and stored as TG (Braun and Severson 1992).

1.2.2. Lipolysis and β-oxidation

Mobilization of fatty acids from TG stores in WAT requires lipolytic enzymes, such as HSL and ATGL. They are being activated under the conditions of energy demand resulting in the release of free fatty acids and glycerol into bloodstream, which are important oxidative fuels for other tissues such as liver (Zimmermann et al. 2004). HSL (also known as LIPE), encoded by the Lipe gene, was the first enzyme discovered that hydrolyzes triglycerides in mammalian adipose tissue. It responds to hormones action, being activated by catecholamines and inhibited by insulin (Carmen and Victor 2006). Although transcription of

Lipe was initially described as specific for adipocytes, subsequent studies have shown that it is expressed and has a role in lipid metabolism in multiple tissues, including liver and macrophages (Yeaman 2004). In turn, ATGL is rate-limiting for the initial step of triacylglycerol (TAG) hydrolysis, generating diacylglycerol (DAG) and fatty acids. It exhibits high substrate specificity for triacylglycerol and is associated with lipid droplets. Its inhibition markedly decreases total adipose acyl-hydrolase activity (Zimmermann et al. 2004).

Unlike PPARγ isoform, PPARα is expressed mainly in BAT and liver and induces the expression of genes involved in mitochondrial and peroxisomal fatty acid oxidation, as well as mitochondrial ketogenesis, such as the acyl-CoA dehydrogenase (Acd), and acyl-coenzyme A oxidase (Acox) (Schoonjans et al. 1996). Moreover, PPARα induces the endocrine hormone fibroblast growth factor 21 (FGF21) in liver in response to fasting, which has been found to stimulate lipolysis in WAT and ketogenesis in liver (Inagaki et al. 2007). PPARα also regulates CPT1, which is involved in the mechanism by which long chain fatty acids are translocated from citosol to mitochondrial matrix to be degraded through β-oxidation (Esser et al. 1993). The regulation of enzymatic activity of CPT1 is mediated by the presence of malonyl-CoA. The formation of the latter is catalyzed by ACC that is expressed in two isoforms, ACC-α and ACC-β. The ACC-α isoform predominates in lipogenic tissues, such as liver and adipose tissue (Kerner and Hoppel 2000).

A member of the retinoid X receptor (RXR) family of nuclear receptors, RXRb, has been linked to lipid metabolism with dual oxidative and lipogenic effects. On the one hand, RXRb heterodimerizes with PPARα and in this way cooperates in the induction of the acyl-CoA oxidase gene, which encodes the rate-limiting enzyme of peroxisomal β-oxidation of fatty acids (Keller et al. 1993). On the other hand, RXR may also induce FASN via formation of LXR/RXR heterodimers binding to their recognition sequences in SREBP1c promoter (Roder et al. 2007).

1.3. Key regulators of energy homeostasis – insulin and leptin function

Peripheral hormones such as leptin and insulin have received much attention as modulators of feeding patterns and energy expenditure at the hypothalamic level, acting as anorexigenic hormones. Circulating concentration of leptin appears to mirror fat cell stores - increasing with overfeeding and decreasing with starvation (Coll et al. 2007). Plasma insulin concentration in turn, depends on peripheral insulin sensitivity, which is related to both total

body fat stores and fat distribution, with visceral fat being a key determinant. However, unlike leptin levels, which are relatively insensitive to acute food intake, insulin secretion increases rapidly after a meal (Stanley et al. 2005).

1.3.1. Role of insulin and leptin in central control of energy homeostasis

As described above, both leptin, the adipocyte-derived hormone, and insulin, the pancreatic β-cell-derived hormone, circulate in the bloodstream in proportion to body fat mass and regulate the activity of neurons present in regions of the brain associated with body weight regulation. In the ARC there are at least two distinct neuronal cell types, namely anabolic and catabolic. Anabolic neurons are those that, when activated, promote an increase in food intake and a decrease in energy expenditure, leading to the storage of energy. In the ARC, these neurons coexpress the orexigenic NPY and AgRP. Catabolic neurons express POMC and CART, and act in opposition to anabolic neurons. Both types of cells respond to insulin and leptin in the opposite manner: NPY/AgRP neurons are inhibited and POMC/CART neurons are activated. ARC neurons project to other key brain areas and are thought to function as primary neurons in a series of neural circuits that regulate food intake and energy expenditure, to maintain energy balance and body fat stores (Figure 1) (Niswender et al. 2004).

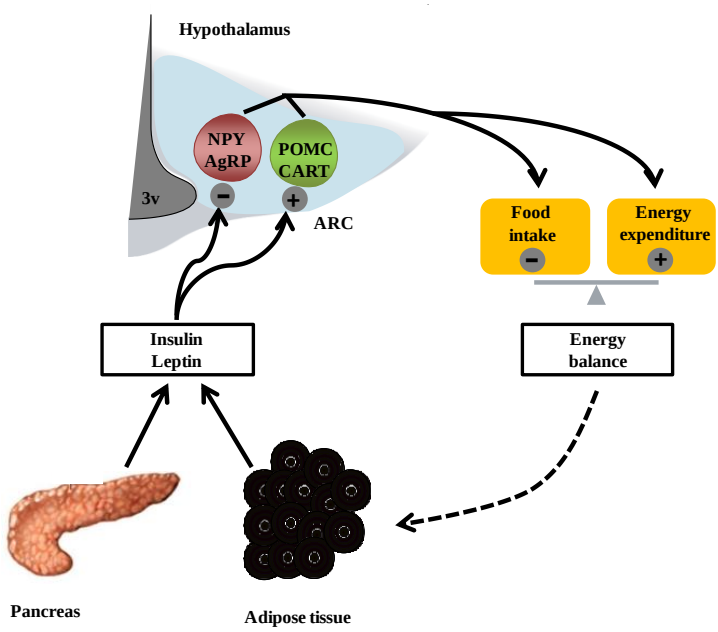


Figure 1. Model of central control of energy homeostasis: role of insulin and leptin. Neurons in the arcuate nucleus (ARC) express insulin and leptin receptors and integrate peripheral signals to maintain energy homeostasis. The ARC contains anabolic neurons coexpressing neuropeptide Y (NPY) and agouti-related protein (AgRP) and catabolic neurons expressing pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). Both types of cell respond to insulin and leptin in the opposite manner: NPY/AgRP neurons are inhibited and POMC/CART neurons are activated. It is thought that this coordinate dual regulation of opposing cell types ultimately regulates complex responses, such as changes in feeding and energy expenditure, to maintain body fat stores; 3v, third ventricle. Adapted from (Niswender et al. 2004).

Insulin and leptin resistance

Human obesity is characterized by hypothalamic resistance to adiposity signals, such as insulin and leptin. Leptin and insulin resistance can be assessed by increased Socs-3 (an inhibitor of cytokine signaling) and reduced Obrb and Insr mRNA levels (Ahren et al. 1999; Levin and Dunn-Meynell 2002). Leptin achieves its control on metabolic processes through its interaction with the leptin receptor (OBR) that appears at least in six isoforms, from OBRa to OBRf, having C-terminal domains with different lengths. Among them, the full-length receptor, OBRb transmits leptin signal through its C-terminal tyrosine residues and associated proteins being crucial for leptin action, and thus, for energy homeostasis (Myers et al. 2008). In leptin resistance state, OBRb content in the hypothalamus has been shown to increase at first but then to decrease in the presence of continuous leptin stimulation (Lin et al. 2000; Fernandez-Galaz et al. 2002), which leads to uncontrolled appetite, although plasma leptin levels are elevated (Shimizu et al. 2007). Cellular insulin resistance in turn, is defined as the alteration of the intracellular propagation of the signals evoked upon activation of the insulin receptor (Fruhbeck 2006).

In addition to the central action, leptin causes peripheral effects, which are mediated through the CNS or through direct actions on target tissues. Thus, hypothalamic leptin stimulates glucose uptake in skeletal muscle, heart, and BAT via the sympathetic nerve and β-adrenergic mechanism (Minokoshi et al. 2012). In turn, the direct action of leptin includes inhibition of insulin secretion and gene expression in pancreatic β-cells (Zhao et al. 1998), as well as stimulation of fatty acid oxidation in adipocytes (Muller et al. 1997). In addition, leptin also exerts direct effects on liver, a major site of glucose metabolism, where it mimics some of the anabolic actions of insulin on this tissue. Leptin has been found to enhance the inhibitory effects of insulin on glycogenolysis (Nemecz et al. 1999), and increases glycogen synthesis in perfused mouse liver (Cohen et al. 1998). Nevertheless, available literature describing the effects of leptin on hepatic glucose metabolism is often equivocal. For example, leptin has been shown both to increase (Nemecz et al. 1999) and decrease (Ceddia et al. 1999) hepatic gluconeogenesis in rats. In turn, peripheral insulin resistance refers to diminished insulin-mediated uptake of glucose principally by skeletal muscle. It depends primarily on the control of GLUT4 glucose transporter expression and translocation to the plasma membrane (Fruhbeck 2006). Insulin promotes glucose disposal in adipose tissue and muscles, and prevents the liver from producing more glucose by inhibition of glycogenolysis and gluconeogenesis. Insulin also controls other important processes such as synthesis and

storage of fat, protein synthesis, cell growth, cell proliferation, survival and differentiation (Fruhbeck 2006). In addition, impaired suppression of hepatic glucose production, which largely accounts for hyperglycaemia and glucose intolerance may lead to hepatic insulin resistance (Fruhbeck 2006).

2. The impact of early life environmental conditions on energy balance control

Unhealthy lifestyle factors, such as a lack of physical activity and excessive calorie intake undoubtedly contribute to the worldwide prevalence of obesity and its related chronic diseases. Despite these key components in the development of obesity, it is widely accepted that factors during perinatal stages of life, such as overnutrition, malnutrition, and gestational diabetes may program the incidences of obesity and its related metabolic pathologies in adult life (Cottrell and Ozanne 2008; Sullivan and Grove 2010). The "fetal origins of adult disease¨ hypothesis, originally put forward by Barker and Osmond in 1986, states that environmental factors, particularly nutrition and maternal health, act in prenatal and early postnatal stage of life to program the risks for the early onset of cardiovascular disease in adult life and premature death (Barker and Osmond 1986; Barker et al. 2002). This hypothesis has been further developed through a worldwide series of epidemiological and experimental studies. It has been evidenced that the perturbation of the early nutritional environment is associated with an increased risk for hypertension, impaired glucose tolerance, type 2 diabetes, insulin resistance, central obesity and the metabolic syndrome in adult life (McMillen and Robinson 2005). In addition, it has been proposed that both maternal and paternal body weight and diet peri-pregnancy are crucial for programming offspring propensity to develop obesity and metabolic dysfunctions in later life (Spencer 2012).

In humans, paternal obesity has been associated with lower birth weight in progeny (Power et al. 2003) and higher adiposity levels in prepubertal girls (Figueroa-Colon et al. 2000). In turn, in animal studies, paternal high-fat diet has been shown to program β-cell dysfunction, which led to impaired insulin secretion and glucose tolerance in female rat offspring (Ng et al. 2010). Despite mentioned paternal influence on offspring phenotype and metabolic function, the maternal influence appears to be stronger than that of the father (Parsons et al. 2001).

Several studies provided evidence that maternal obesity at the start of pregnancy, excessive pregnancy weight gain and gestational diabetes increase the risk of obesity and metabolic syndrome in childhood (Ruager-Martin et al. 2010; Boney et al. 2005; Gaillard et al. 2013). However, not only the incidence of women being overweight or obese, but also maternal

overfeeding, and particularly consumption of high-fat and junk food diets during pregnancy, exerts detrimental effects on offspring health (Muhlhausler et al. 2006; Srinivasan et al. 2006; Bayol et al. 2007). These results indicate that intake of these foods and diet by female rats malprogrammed the offspring for glucose intolerance and increased body weight in adulthood (Srinivasan et al. 2006), altered the responses of the central appetite regulatory system to signals of increased adiposity after birth (Muhlhausler et al. 2006) and led to the development of an exacerbated preferences for fatty, sugary and salty foods in later life (Bayol et al. 2007).

The issue of maternal obesity and overnutrition is of high importance regarding the current global obesity epidemic and trends for the consumption of palatable foods. However, the main field of 'developmental origins of adult health and disease' has not been focused on the increased foetal nutrient supply, foetal overgrowth and infant fatness, but, has been concentrated on the lasting effects of poor foetal nutrition.

Being born small for gestational age or being born to undernourished mothers during pregnancy exert harmful effects on the health of offspring. Poor gestational nutrition provokes metabolic adaptations in foetus that increase survival of the foetus under these detrimental conditions, but, also the likelihood of developing obesity and other chronic disorders, particularly under an obesogenic environment (Hales and Barker 2001). Over the past three decades considerable number of epidemiological human studies addressing the association between poor foetal growth and health consequences in later life has been published (Roseboom et al. 2000b; Stanner et al. 1997). Moreover, a wealth of animal studies has also been conducted to gain further inside into the mechanisms linking this prenatal condition, induced by maternal dietary restriction by calorie, protein or specific nutrient deficiency, and perturbations in the homeostatic control of energy balance (Palou et al. 2010a; Ikenasio-Thorpe et al. 2007; Venu et al. 2004).

Nowadays, the rate of prevalence of childhood overweight and obesity phenomenon is higher in developing than in developed regions. According to World Health Organization (WHO), more than 30 million overweight children are living in developing countries and 10 million in developed countries (WHO 2005). Although, the presence of maternal undernutrition during gestation does not appear to account for the increasing prevalence of obesity in developed societies nowadays, it might explain the increasing prevalence of obesity-related pathologies among people in developing countries where maternal malnutrition remains common (Rasmussen 2001). Moreover, fetal undernutrition may also arise as a result of deficiency

caused by maternal failure of complete absorption of food components (McArdle and Ashworth 1999) and differences in seasonal food availability (Rasmussen 2001). The identification of risk factors is the key to prevention, thus, might be of high importance to develop studies that help to elucidate the programming mechanisms of these prenatal conditions.

As far as contribution of the environment during perinatal stage of life is concerned, not only is the nutrition during pregnancy crucial but also nutritional conditions during postnatal period may determine later obesity.

The importance of this period is also evidenced by "catch-up growth" phenomenon, as subjects who were small or thin at birth and then showed rapid childhood growth showed the greatest risk for disease in adulthood (Eriksson et al. 1999; Crowther et al. 1998). However, even in offspring born at normal weight, early postnatal overnutrition predisposed rats for the development of obesity, diabetes and also cardiovascular risk in later life (Plagemann et al. 1992). Nevertheless, during the immediate postnatal period after delivery, maternal milk is the only source of food for the suckled offspring. In this regard, breastfeeding, as well as breast milk leptin, have been described as a protective factors against the development of obesity (von Kries et al. 1999; Miralles et al. 2006). Thus, owing to remarkable role of maternal milk during this period, the issue of maternal nutrition during lactation might also be of high importance. However, unlike the number of studies exploring the effects of foetal undernutrition, the consequences of maternal undernutrition or calorie-restriction during the lactation period have received less attention. Despite the studies that address the effects of this postnatal condition on maternal milk production (Dusdieker et al. 1994), the lasting consequences on the health outcomes in offspring have been poorly explored.

All in all, nutrition in early life influences offspring likelihood of getting fat later in life. In the studies, which investigate the effects of nutrition in early life three distinct hypotheses have been considered (Figure 2) (Martorell et al. 2001): 1) overnutrition increases the risk of later fatness; 2) at the other extreme, undernutrition is also associated with increased risk of fatness; and 3) optimal nutrition during infancy, represented by breastfeeding, is protective of future obesity

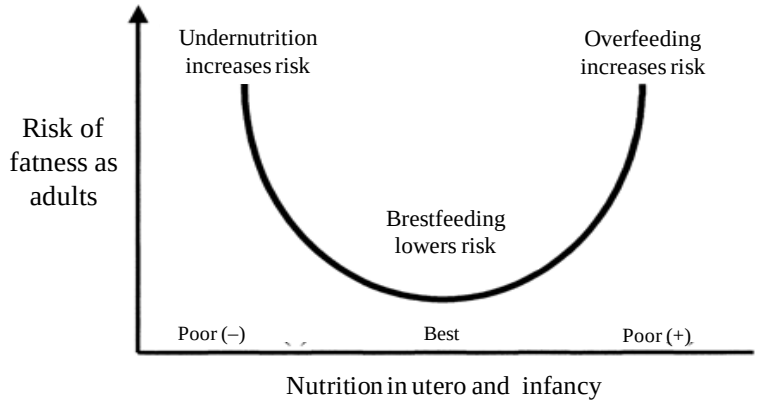


Figure 2. Hypothesis about early childhood nutrition and risk of fatness in adulthood (Martorell et al., 2001).

2.1. Programming effects of maternal food restriction during pregnancy on the offspring susceptibility to obesity

Gestation is one of the most important developmental stages in which maternal health, nutrition and well-being plays remarkable role. It is widely established that maternal prenatal undernutrition, as well as low birth weight, have been described to affect offspring metabolism, and have been associated with obesity and metabolic syndrome in adulthood both in humans and rats (Ravelli et al. 1976; Grino 2005; Jones et al. 1984). The hypothesis of ¨thrifty phenotype¨ coined by Hales and Barker in 1992 proposes that poor foetal nutrition results in metabolic adaptations that program to accumulate energy, thereby, maximize chances of surviving postnatally in conditions of ongoing deprivation. However, when the postnatal environment provides more abundant nutrition, these adaptations become detrimental, leading to the increased risk of developing type 2 diabetes and the metabolic syndrome (Hales and Barker 2001).

2.1.1. Outcomes derived from epidemiological studies

The renowned evidence in scientific literature supporting the proposal of the thrifty phenotype hypothesis comes from an epidemiological study on the consequences of Dutch famine, which took place in Holland near the end of World War II. This study has demonstrated that young adult males born to mothers who underwent malnutrition as a result of this famine during the first two trimesters of gestation (but not the last trimester) were more likely to be obese in adult life than those born from mothers who did not experience poor nutrition (Ravelli et al. 1976). Prenatal undernutrition had only a small impact on birth weight of foetally undernourished infants, but in the sixth decade of life they exhibited

alterations in plasma lipid profile (Roseboom et al. 2000a), higher prevalence of coronary heart disease (Roseboom et al. 2000b), and renal dysfunction (Painter et al. 2005). However, another epidemiological study in humans has revealed that exposition to poor foetal nutrition due to famine occurred during the siege of Leningrad (1941-44) had no association with the risk of adult obesity (Stanner et al. 1997). This might be due to the fact, that unlike in Holland, where a sudden famine lasted shortly (5 months) and was followed by adequate nutrition, the siege of Leningrad lasted significantly longer (28 months) and living conditions remained poor after this period (Spencer 2012). In fact, effects of poor foetal nutrition on the incidence of coronary heart disease, type 2 diabetes and hypertension could be attributed to low birth weight and/or catch-up growth in infancy and rapid weight gain in adolescence due to improved postnatal nutrition (Eriksson et al. 1999; Barker et al. 2002).

2.1.2. Outcomes derived from animal experimentations

Following the findings in human studies, a number of animal studies have been developed leading toward better understanding the issue of the association between maternal prenatal undernutrition and obese phenotype in offspring in later life. Notably, changes in maternal nutrition can lead to altered long-term health effects in offspring independently of size at birth (Harding et al. 2011). By now, the most described animal models are those in which foetal undernutrition has been induced by global calorie restriction (from severe to mild) or reduction of dietary protein content in maternal diet. However, different findings have been obtained depending on gender and type, duration and severity of restriction (Pico et al. 2012). Some studies have shown that male rats of 50% calorie-restricted dams (during the first two weeks of pregnancy) exhibited higher food intake and became obese after 5 weeks of age (Jones and Friedman 1982). Similar treatment during pregnancy in dams resulted in increased fat accumulation from 1 to 53 days of age in female rat offspring, despite no changes in food intake (Anguita et al. 1993). Moderate gestational calorie restriction of 30% (during the entire period or the first 2 weeks of pregnancy) has also been shown to cause hyperphagia and higher fat accumulation in the offspring, particularly under hypercaloric diets, without affecting body weight (Vickers et al. 2000; Vickers et al. 2005). A more moderate calorie restriction of 20% during the first 12 days of gestation caused hyperphagia in the adult male and female offspring, that was associated with higher body weight and body fat content in males but not in females (Palou et al. 2010a).

Studies performed in animal models of maternal undernutrition during gestation have also provided evidences for detrimental effects of this condition on food preferences and physical activity in the offspring, which may contribute to the obese phenotype of adult offspring (Bellinger et al. 2004; Palou et al. 2010a; Vickers et al. 2003; Bellinger et al. 2006). Both issues might be of high importance for humans, owing to lifestyle in developed countries favoring high intake of palatable foods rich in energy, fat and sugar combined with a lack of exercise. It has been shown that male and female offspring of dams fed with 50% protein-restricted diet throughout gestation displayed preferences for energy-dense foods in later life, which was accompanied by hyperphagia, and higher body weight and fat content than controls (Bellinger et al. 2004). Moreover, male, but not female offspring, of 20% calorie-restricted dams during the first half of pregnancy showed greater preference for fat-rich food than their controls, which was also accompanied by higher food intake and body weight when exposed to high-fat diet (Palou et al. 2010a). As far as physical activity is concerned, maternal prenatal undernutrition, induced by both calorie and protein restriction, was able to affect locomotor activity of adult offspring (Bellinger et al. 2006; Vickers et al. 2003). For example, offspring from 30% calorie-restricted mothers during pregnancy showed alterations in voluntary locomotor activity, independently of postnatal nutrition, although sedentary behavior was exacerbated by postnatal hypercaloric nutrition (Vickers et al. 2003). Thus, programmed changes in food preferences and activity might be possible mechanisms responsible for hyperphagia and overweight often observed in adult offspring of undernourished dams during pregnancy.

2.1.3. Mechanisms linking maternal food restriction during pregnancy and offspring susceptibility to obesity

Although, the mechanisms by which prenatal food restriction programs likelihood of developing obesity in adult offspring have not been clearly elucidated, there are in scientific literature evidences for possible mechanisms involved. The mechanisms proposed include permanent perturbations in the structure of key organs, alterations of circulating hormones levels and epigenetic modifications (Pico et al. 2012). This section addresses the effects of maternal food restriction during pregnancy on offspring hypothalamic structure and function, as well as on sympathetic innervation in peripheral key organs.

Permanent perturbations in the hypothalamic structure and function

The brain is particularly sensitive during early life and undergoes alterations in response to nutritional environment. Maternal undernutrition programs adjustments in offspring hypothalamic nuclei that regulate energy homeostasis, with the alterations seen already at weaning at different levels: size of the hypothalamic areas and number of neurons found in each area, the interconnection that the neurons form between the different areas, the types of neurons present in each specific area and the expression of receptors for hormones and neuropeptides in these neurons (Pico et al. 2012).

Studies in animal models have evidenced that nutritional manipulations in maternal diet during perinatal period, such as protein or calorie restriction (Plagemann et al. 2000; Ikenasio-Thorpe et al. 2007; Delahaye et al. 2008; Garcia et al. 2010) modify hypothalamic structure, which was usually accompanied by alterations in the mRNA expression levels of the appetite-regulatory neuropeptides. In particular, offspring of dams fed a low-protein diet during gestation and lactation displayed a reduction of NPY neurons in the ARC as well as a greater relative volume of ventromedial hypothalamic nucleus (VMH) and a lower absolute volume of the PVN (Plagemann et al. 2000). Perinatal 50% food restriction during perinatal period disturbed hypothalamic nerve fibres projections from the ARC to the PVN and reduced the expression of POMC, the precursor of anorexigenic neuropeptide αMSH in neonate rats (Delahaye et al. 2008). Gestational caloric restriction of 30% altered hypothalamic expression of Pomc, as well as orexigenic neuropeptides, such as NPY and AgRP in offspring postnatally exposed to high-fat diet (Ikenasio-Thorpe et al. 2007). In turn, more moderate maternal caloric restriction (20%) during pregnancy perturbed in weaned rats hypothalamic ARC structure and function, by decreasing the exhibition of total number of arcuate cells, as well as its NPY- and αMSH-neurons, accompanied by lower expression levels of Npy and Agrp (Garcia et al. 2010). Although, the effects of maternal undernutrition during pregnancy depend on the type, quantity and duration of the restriction, the effects of these maternal nutritional conditions during early life are mostly manifested by a dominance of the orexigenic versus anorexigenic drive in the offspring, which program the likelihood for obesity and other alterations in later life (Pico et al. 2012).

Reduced expression levels of hypothalamic leptin and insulin receptors, together with altered signalling of these hormones, has also been proposed to contribute to later alterations associated to maternal undernutrition during gestation (Pico et al. 2012). In this regard, lower

expression levels of leptin and insulin receptors and an impaired response of the hypothalamus to fed/fasting conditions were observed in 25-day-old offspring of 20% calorie-restricted dams during gestation (Garcia et al., 2010). Decreased expression levels of hypothalamic leptin receptor were also observed in adult offspring of 70% calorie-restricted dams throughout gestation, together with an altered response of leptin and insulin receptors to fasting conditions (Breton et al. 2009).

Leptin has been shown to play an important role during the critical windows of developmental plasticity, programming hypothalamic circuits formation, which in rodents coincides with an transiently increase in plasma leptin levels during the short postnatal period, the so-called neonatal leptin surge (Bouret and Simerly 2006). Alterations in leptin levels during this period result in permanent structural consequences affecting development of hypothalamic orexigenic and anorexigenic centers. In fact, maternal perinatal 50% food restriction has been shown to reduce drastically the postnatal surge of plasma leptin, disturbing the hypothalamic wiring in male rat pups (Delahaye et al. 2008). A premature leptin peak, as occurring in mice with severe fetal undernutrition (Yura et al. 2005) resulted in increased nerve terminal densities of NPY and CART in PVN. These results may give another clue about the association between maternal undernutrition during early life and adverse health outcomes in the offspring contributing to the development of metabolic disorders in adulthood.

Perturbations in the sympathetic nervous system

The development of the peripheral structures of the nervous system in offspring has also been shown to be affected by nutritional manipulations of maternal diet during gestation. Specifically, 50% maternal calorie restriction during the last two weeks of pregnancy affected the density of the sympathetic innervation and enteric neuron number in the jejunum of adult rats, and thus disturbing the control of gut motility (Santer and Conboy 1990). Furthermore, 20% moderate maternal calorie restriction during the first part of gestation altered sympathetic innervations of WAT, which was accompanied by development of hyperplasia in this tissue in the adult male offspring exposed to high-fat diet (Garcia et al. 2011). The same model of maternal undernutrition during pregnancy resulted in the decreased adrenergic sympathetic drive to the stomach in offspring at juvenile age, which has been proposed to account for the reduction of circulating ghrelin levels occurring in young and adult offspring (Garcia et al. 2013). Thus, these perturbations in the structure of SNS might program for

lasting adverse effects of these prenatal conditions on the control of energy metabolism in offspring.

All in all, human epidemiological studies and experimentations on animal models have evidenced for foetal programming of adult chronic diseases, mostly including obesity, cardiovascular disease, hypertension, insulin resistance and type 2 diabetes. In addition, poor foetal and/or low birth weight has also been linked with alterations in other systems and functions leading to non-chronic diseases, such as infections, reduction of bone mass, cognitive ability and immunity, as well as autoimmune and renal disease (Delisle and World Health Organization 2001). These associations may also support the importance of promoting adequate growth in early life.

2.2. Programming effects of maternal food restriction during lactation on the offspring susceptibility to obesity

Unlike considerable number of studies focused on exploration of food restriction during pregnancy, the lasting consequences of undernutrition during the lactation period have received less attention. Moreover, among limited number of studies that were performed in women during lactation, most has been mainly focused on the determination of milk composition and its volume, but the lasting effects of this condition on the children's health have been poorly explored. In turn, reports from studies carried out in various types of animal models are not consistent, as both positive (Palou et al. 2010b) and negative (Boxwell et al. 1995) impacts of maternal undernutrition during lactation on the health outcomes in progeny have been observed.

2.2.1. Effects of maternal food restriction during lactation on milk composition and volume

In general, it appears that malnutrition during lactation has little if any effect on protein concentration in human (Lonnerdal 1986) and rat (Palou et al. 2010b) milk, as well as on proteins proportion in malnourished rat dams (Zubieta and Lonnerdal 2006), but it does has an impact on fatty acid concentrations (Lonnerdal 1986). There are different reports from studies investigating the effects of maternal malnutrition on milk production. On the one hand it has been shown that maternal isocaloric low protein diet in lactating rats reduces milk quantity (Bautista et al. 2008). On the other hand, modest or a short-term weight loss by breast-feeding women does not adversely affect either amount or energy output of milk consumed by their infants (Dusdieker et al. 1994; McCrory et al. 1999). Similarly, no

significant effect on milk output was found in an study carried out in non-human primates submitted to a moderate calorie restriction of 20%, but it was significantly reduced when calorie restriction was raised up to 40% calorie restriction (Roberts et al. 1985). Thus, the effects of maternal dietary food restriction on maternal milk production still remain to be established, particularly regarding investigations of the long-term effects of this condition in human studies, which are difficult to perform.

2.2.2. Effects of maternal food restriction during lactation on health outcomes in offspring

Most of the animal models designed to investigate the effects of undernutrition during lactation period on the health outcomes in offspring have been performed by manipulations in maternal diet, inducing global calorie restriction, reduction of dietary protein or micronutrients content (Fagundes et al. 2007; de Souza Caldeira and Moura 2000; Boxwell et al. 1995), or by direct manipulations in offspring, such as increasing the litter size (Remmers et al. 2008). Similarly to the results obtained from studies regarding undernutrition during pregnancy, also in the case of undernutrition during lactation, the health outcomes obtained in offspring depend on the type and severity of restriction. Regarding the phenotypic traits in offspring, in rats, malnutrition produced by severe protein restriction of 65% (Fagundes et al. 2007) or by removing of proteins from maternal diet (de Souza Caldeira and Moura 2000) during lactation has been associated in the adult offspring with reduced body weight, despite no changes in food intake. In turn, maternal isocaloric low protein diet of 50% during lactation resulted both in decreased body weight and food intake in the offspring (Zambrano et al. 2006). Similarly, other energy restriction models obtained by severe global food restriction of 67% in maternal diet during the lactation period (Boxwell et al. 1995) or by increasing the litter size (Remmers et al. 2008) were also associated with lower body weight and energy intake, as well as lower body length in adult offspring. Unlike the models of severe nutritional restriction during lactation, moderate maternal calorie restriction of 30% during this period has been shown to result in lower body weight and fat content in both male and female offspring, without any changes in body length (Palou et al. 2010b). The lower body weight of these animals was associated with lower food intake, which was maintained from weaning to adulthood. In addition, these moderate conditions also programmed food preferences, as female offspring displayed a lower preference for fat-rich food when exposed to high-fat diet feeding (Palou et al. 2010b).

Despite described changes in anthropometric parameters and food intake, severe maternal malnutrition during lactation also affected circulating thyroid hormones, insulin and leptin levels in the offspring. Protein restriction in maternal diet during lactation resulted in increased thyroid function showed by higher thyroid uptake and thyroid hormones serum concentration in the offspring of those dams compared to controls. The hyperthyroidism observed in the offspring could be the reason of low body weight observed in those animals (Passos et al. 2002). Unlike in the models of malnutrition during pregnancy, food restriction during lactation seems to improve insulin sensitivity in the offspring. Thus, submission of dams to isocaloric low protein diet during lactation improved insulin sensitivity in the offspring at juvenile age, as suggested by lower concentrations of insulin and upregulation of key proteins involved in insulin/insulin-like growth factor 1 (IGF-1) signaling pathway in kidney (Martin-Gronert et al. 2008). Another study conducted on the adult offspring of 50% protein-restricted dams during lactation also showed some evidences of increased insulin sensitivity in those animals (Zambrano et al. 2006). There are different findings from studies regarding circulating leptin levels. On the one hand, submission of dams to severe energy restriction (Boxwell et al. 1995) or to an increment of litter size (Remmers et al. 2008) resulted in diminishment of leptin levels in animals at juvenile age, as well as in adulthood (Remmers et al. 2008). On the other hand, severe protein restriction of 65% in lactating dams significantly decreased leptin serum concentration in the offspring on day 12 of life, but increased its levels already at the age of 21 days (Teixeira et al. 2002). In turn, the model of moderate maternal calorie restriction of 30% resulted in certain protection on the adult offspring against the increase of insulin and leptin plasma levels occurring in their controls under HF diet feeding at the age of 20 (Palou et al. 2010b) and 28 weeks (Palou et al. 2011b). These adjustments were accompanied by improvement of their sensitivity to insulin and leptin signalling in a key tissues involved in the control of energy balance, but in a manner that was different between male and female animals (Palou et al. 2011b).

All in all, results from animal studies have shown that the effects of maternal malnutrition during lactation on the health outcomes in offspring, and particularly on the propensity to obesity in later life, differ depending on the type and severity of the restriction. Whereas moderate food restriction in lactating dams seems to improve metabolic health of offspring (Palou et al. 2010b; Palou et al. 2011b), severe food restriction in lactating dams (Boxwell et al. 1995)or a diminishment of the total intake of milk by increasing the litter size (Remmers

et al. 2008) may affect normal development of animals. However, the mechanisms that underlie these alterations are still poorly characterized.

2.3. Beneficial effects of breastfeeding on health outcomes in infants

The term lactation refers to the process of milk production, the development and function of the mammary gland, and the period of time when a mother lactates to provide neonates with the nutrients they need for healthy growth and development. This process is the quintessential characteristic of mammals, as it occurs with all of female mammals after delivery, and plays a central role in mammalian reproduction and development.

In animals the length of lactation varies within and among species. It usually lasts until some permanent teeth erupt, although it may be limited by many factors such as insufficient milk production. In humans the process of feeding of an infant is called breastfeeding. Its prevalence and duration have lessened worldwide for a variety of social, economic and cultural reasons. WHO in collaboration with The United Nations Children's Fund (UNICEF) made global recommendations of exclusive breastfeeding of infants up to 6 months of age. However, it may also be continued thereafter along with appropriate complementary foods up to two years of age or beyond (WHO 2014). Although, the composition of milk varies among women and depends on stage of lactation, breast milk provides almost all the necessary nutrients, growth factors and immunological components for a proper growth and development of infants (Leung and Sauve 2005).

Despite benefits for maternal health, enhancing mother-infant bonding and basal nutritive function of maternal milk, breastfeeding has been reported to bring much more advantages for infants. Exclusive breastfeeding has been associated with reduction of incidences and severity of infections in infants, prevention of allergies, prevention of certain gastrointestinal diseases and sudden infant death syndrome, as well as possible enhancement of cognitive development (Leung and Sauve 2005). Moreover, there exist hard evidence indicating that breastfeeding reduces the risk of childhood obesity (Grummer-Strawn and Mei 2004; Toschke et al. 2002), type 1 diabetes mellitus (Gerstein and VanderMeulen 1996) and protects against high blood pressure in later life (Martin et al. 2004). Few studies did not confirm the negative association between being breast-fed and being obese as a child (Hediger et al. 2001; Wadsworth et al. 1999), but they also lacked information on the exclusivity of breastfeeding (Dewey 2003).

Several different hypotheses have been proposed to explain preventive effects of breastfeeding on obesity risk. Studies performed in healthy infants fed on demand indicated that physiological feeding patterns may play important role in this matter, as breast-fed infants ingest more frequently and lower feeding volumes than formula-fed infants (Sievers et al. 2002). Nevertheless, since maternal milk contents fundamentally differ from formula milk contents, it suggests that milk composition could have primary responsibility for programming of a lean phenotype (von Kries et al. 1999). In this regard, it has been shown that infants receiving the formula consumed more total energy and up to 66-70% more proteins than breast-fed infants (Heinig et al. 1993). The higher protein content of infant formula milk might be responsible for the increased growth rate and adiposity during the influential period of infancy of formula-fed infants (Oddy 2012; Koletzko et al. 2009). In addition, breast milk contains hormones, such as ghrelin, leptin and adiponectin, which have a role in energy balance regulation (Savino and Liguori 2008). Among them, the role of hormone leptin during lactation has received particular interest (Palou and Pico 2009).

2.3.1. Leptin and its role during lactation

Leptin was originally thought to be secreted only by adipocytes and act on the brain to regulate food intake and body weight. Later on, leptin was discovered to be also produced by the placenta (Masuzaki), the stomach (Bado et al. 1998; Cinti et al. 2000), the skeletal muscle (Wang et al. 1998) and mammary epithelial cells (Smith-Kirwin et al. 1998). These findings prompted the discovery of new functions of this hormone in the body. Thus, leptin has been found to play role in reproduction (Clarke and Henry 1999), and in the regulation of fetal growth and development due to placental production of leptin and its expression in fetal organs (Ashworth et al. 2000). Leptin has also been reported to affect the cardiovascular and renal changes associated with obesity (Haynes et al. 1997). The expression of leptin in stomach may also function in the short-term system to control feeding behavior (Cinti et al. 2000). Moreover, its action in a distinct region of small intestine was associated with nutritional absorption and lipid uptake, suggesting that leptin can affect fat accumulation, metabolism, and energy homeostasis that is in part independent of the regulation of food intake (Morton et al. 1998).

Milk-borne leptin

The presence of leptin in human breast milk was first described by Casabiell and co-workers in 1997 (Casabiell et al. 1997). Leptin in maternal milk comes from the mammary epithelial cells, from which it is secreted in milk fat globules that partition into the lipid portion of breast milk (Smith-Kirwin et al. 1998). The second factor that contributes to the content of leptin in milk is maternal circulation (Savino and Liguori 2008), as milk leptin levels are positively correlated with maternal plasma leptin levels during the entire lactation period (Houseknecht et al. 1997; Casabiell et al. 1997). Leptin ingested from maternal milk can be absorbed further by the immature stomach of suckled rats and transferred to the their bloodstream (Casabiell et al. 1997; Sanchez et al. 2005), exerting many biological effects in the organism of neonates. In fact, maternal milk is the principal source of leptin for infants during the first months of life, at a time when the adipose tissue is still immature (Palou and Pico 2009).

Available reports about the concentrations of milk leptin vary strongly according to species, period of lactation, milk fractions and the assay methods used when preparing sample for analysis (Bonnet et al. 2002). Thus the concentrations of milk leptin in humans varies from 73.2 ± 13.8 ng/ml (Smith-Kirwin et al. 1998) to 3.4 ± 1.0 ng/ml (Ucar et al. 2000) in whole milk samples and from 1.5 ± 0.9 ng/ml (Houseknecht et al. 1997) to 0.2 ± 0.1 ng/ml (Lonnerdal and Havel 2000) in skim milk. However, it has been evidenced that, unlike maternal milk, leptin is not present in infant formulas (O'Connor et al. 2003). In fact, breast-fed infants display higher plasma leptin levels than formula-fed infants during the first months of life (Savino et al. 2002). Thus, milk-borne leptin might be this specific compound, or at least one of them, responsible for lower risk of obesity development in breast-fed infants than in formula-fed infants. In this sense, a study in nonobese women who breast-fed their infants for at least 6 months has evidenced a negative association between milk-borne maternal leptin levels and body weight gain of infants during the first 2 years of life (Miralles et al. 2006). This negative association has also been found between leptin concentration in mature milk and BMI increase of infants during the first month of life (Doneray et al. 2009), and between breast milk leptin levels and infant weight gain over 6 months of lactation (Schuster et al. 2011). Thus, results from these studies evidence for protective effects of milk-borne maternal leptin against excess weight gain in infants.

Neurotrophic role of leptin

In addition to the role of leptin in the regulation of energy homeostasis in adults, in neonates, leptin has been shown to play a crucial neurotrophic role programming hypothalamic circuits formation during a postnatal short period, particularly nerves projections from ARC to the other hypothalamic areas (Figure 3) (Bouret and Simerly 2006). In rodents, these feeding circuits primarily form during neonatal life and remain structurally and functionally immature until 3 weeks of life (Bouret and Simerly 2006). Neurodevelopment action of leptin appears to be restricted to the critical windows of developmental plasticity, which coincides with an increase in plasma leptin levels, the so-called leptin surge (Bouret and Simerly 2006) (see also section 2.1.3). As originally described by Ahima *et al*., in normally developing rodents there is an increase in circulating levels of leptin between postnatal days 4-16 (Ahima et al. 1998). Disruption or lack of leptin during this period in rodents has been demonstrated to alter the normal postnatal developmental pattern of neural projection in the hypothalamus (Bouret et al. 2004) and to have lasting consequences by impairing the capacity to respond to leptin in adulthood, thus predisposing animals to obesity (Attig et al. 2008; Pico et al. 2011).

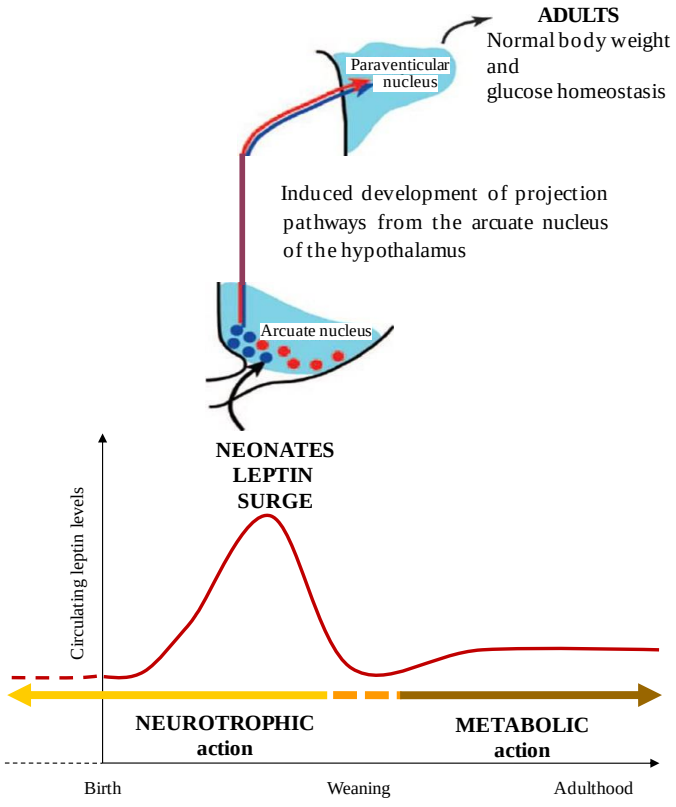


Figure 3. Leptin's actions during neonatal period on the development of hypothalamic feeding circuits in rodents. In addition to its regulatory role in adults, leptin also acts as a neurotrophic signal that directs key developmental events in the same hypothalamic circuits that convey leptin signals to brain regions regulating food intake and body weight. The developmental activity of leptin on projections from the arcuate nucleus appears to be restricted to a neonatal window that corresponds to a period of elevated leptin secretion. Plasma leptin levels increase dramatically during the second week of postnatal life, when leptin fails to alter food intake or body weight, and drop to adult-like levels at weaning. Alteration of the neonatal leptin surge has long-term and irreversible consequences on feeding and metabolism. Adapted from (Bouret and Simerly 2006).

Effects of exogenous leptin treatment during lactation on health outcomes in offspring

Animal research addressing the effects of exogenous leptin treatment to neonate rats provided additional evidences for the essential role of leptin during early life in programming energy metabolism and the propensity to obesity (Pico et al. 2007; Vickers et al. 2005; de Oliveira et al. 2002). The fact that opposite effects were shown in various studies may be attributed to the leptin administration technique, its doses, the treatment duration, different time points of leptin administration, as well as to the nutritional status of pups. Thus, subcutaneous leptin injections at pharmacological doses to neonate rats during the first 10 d of lactation has been shown to program for higher body weight and food intake (de Oliveira et al. 2002) as well as hyperleptinaemia and hyperinsulinaemia in adult rats (Toste et al. 2006). Notably, studies addressing the evaluation of the outcomes of early postnatal leptin supplementation at physiological doses to rats evidenced for protective effects of this treatment against obesity and other metabolic alterations in adulthood. Specifically, daily leptin oral administration throughout lactation to neonate rats born to adequate nourished dams has been shown to protect against age-related increases in body weight, particularly associated with feeding of a high-fat diet (Pico et al. 2007). Leptin-treated offspring displayed improved insulin and leptin sensitivity (Pico et al. 2007; Sanchez et al. 2008), lower preference for fat-rich food in adulthood (Sanchez et al. 2008), as well as improved the metabolic response of adipose tissue to a high-fat diet (Priego et al. 2010). This was associated with hypothalamic changes in the expression of genes involved in the central action of leptin (Pico et al. 2007), and in the methylation of an appetite-related gene in this tissue of animals fed HF diet (Palou et al. 2011a).

Leptin treatment during early life has also been found to have the capacity to reverse developmental malprogramming effects. Daily intraperitoneal injections of pharmacological doses of leptin between postnatal day 4 and 12 in leptin deficient mice (ob/ob) has been shown to rescue the development of disrupted ARC projections (Bouret et al. 2004). Vickers *et al.* evidenced that daily subcutaneous injection of leptin from postnatal day 3-13 in female rats born to undernourished mothers during pregnancy normalized calorie intake, locomotor activity, body weight, fat mass, and fasting plasma glucose, insulin and leptin concentrations in adulthood (Vickers et al. 2005). Furthermore, the same treatment with leptin to male pups conferred protection against development of programmed phenotype, by transient slowing weight gain, as well as decreasing fat mass and plasma insulin concentrations in adulthood (Vickers et al. 2008). Overall, these findings point out that leptin may be recognized as an

essential compound during lactation in imprinting healthier metabolic responses in later life (Palou and Pico 2009), and may also be worth considering when searching for strategies to reverse programmed susceptibility to obesity acquired by fetal undernutrition (Pico et al. 2011; Vickers and Sloboda 2012).

3. Biomarkers as indicators of physiological processes related to obesity and its associated chronic diseases

The challenge for biomedical studies is to discover and elucidate the relationship between health and disease on the one hand, and nutrition and drugs on the other hand. Development of biomarkers may help to guide these issues and recognize frontiers between them. A biomarker is defined by the National Institutes of Health (NIH) as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [(Biomarkers Definitions Working Group 2001), p. 89]. A biomarker may be measured on a biosample (blood, urine, or tissue), it may be a recording obtained from a person (blood pressure, electrocardiography), or it may be an imaging test (echocardiogram, computed tomography scan). Biomarkers can indicate a variety of health or disease characteristics, including the level or type of exposure to an environmental factor, genetic susceptibility, genetic responses to exposures, markers of subclinical or clinical disease, or indicators of response to therapy (Vasan 2006).

There are two major types of biomarkers used in medicine (Mayeux 2004): biomarkers of exposure, which are used in risk prediction, and biomarkers of disease, which are used in screening and diagnosis and monitoring of disease progression. Most biomarkers of exposure measure antecedent factors thought to modify (increase or decrease) the risk of developing the disease. They reflect the sensitivity of a subject to a disease, hence improving early identification of susceptible individuals. As an example of exposure or antecedent biomarker, may serve the surveys of plasma cholesterol levels in order to identify the risk of a heart disease. A diagnostic biomarker is haemoglobin A1C in the blood which is used as standard tool to determine blood sugar control for patients with diabetes, indicating about the disease progression.

The frontiers between being health and being ill are not established. It is now well recognized that nutrition can contribute to promote health, reduce the risk and severity of illnesses (Palou et al. 2009), although it is often suitable for interventions until certain limits in the disease

pathway, as it cannot cure. However, foods contain components that can have both positive and negative effects, as they are a mixture of different components that exert a combined effect on our health. The degree to which foods influence the balance between healthy and disease states may depend on age, gender, physiological or pathological conditions, individual genetic constitution and lifestyle history. Therefore, the evaluation of both positive (benefits) and negative (risks) effects of foods and food components on health is needed (Palou et al. 2009). Moreover, the development of food-based strategies is, however, severely hampered by the lack of predictive biomarkers, especially those that are accessible and quantify health (van Ommen et al. 2009). Most of currently existing biomarkers are those developed for disease and are used in medicine as diagnostic tools. Biomarkers derived from disease processes, such as those identified in obese subjects (Fung et al. 2001) or in animal models of obesity, might not serve to assess the potential benefits of bioactive compounds or changes in dietary habits aiming to decrease the risk of obesity and related metabolic alterations in healthy or at-risk individuals. Thus, besides of biomarkers of disease, it would be of particular interest to identify early biomarkers of health, associated with dynamic changes against nutritional or environmental stressors to maintain homeostasis (Figure 4).

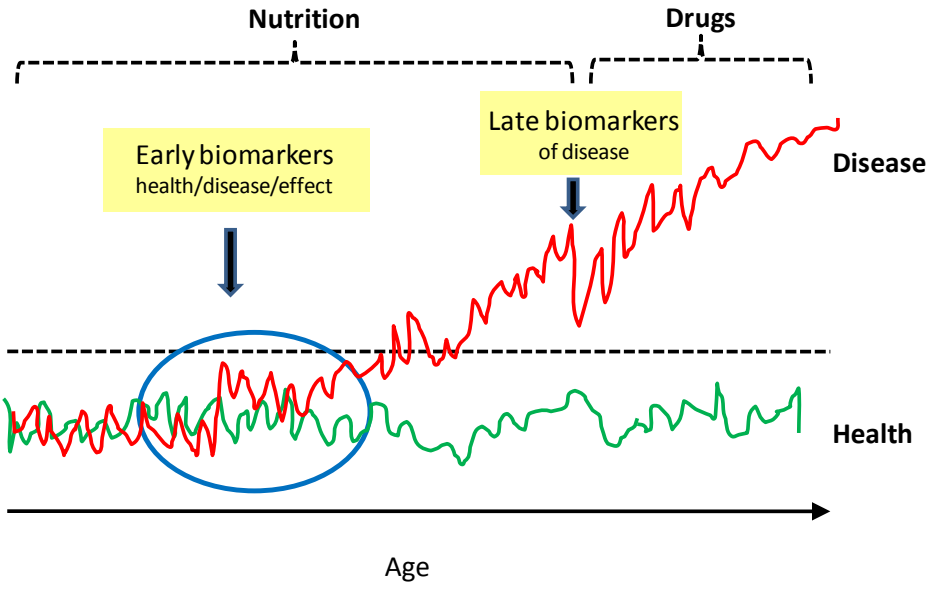


Figure 4. New concepts of biomarkers. Early biomarkers of health/disease are needed to efficiently apply dietary strategies to prevent disease or to recover homeostasis. These biomarkers may also help to distinguish frontiers between health and disease. Therefore, there is growing need for the development of both multiple novel disease biomarkers, beyond the prediction of traditional metabolic risk factors, and biomarkers that may quantify health. Most of currently existing biomarkers are those that are used to diagnose different stages of disease, which are mainly based on changes in unique parameter. However, it would be of particular interest to identify early biomarkers with implications in health, which are associated with dynamic changes against nutritional or environmental stressors to maintain homeostasis. Nutrition and pharma are complementary approaches. Nutrition plays a main role to maintain health and in the early stages of disease. However, it has limited effect in advanced stages of disease. In that case drugs play the main role as a therapeutic agent.

3.1. Nutrigenomic approaches for biomarkers development

Biomarkers widely used in nutritional and clinical studies that address the effect of dietary interventions on the risk of obesity and its co-morbidities development (usually cardiovascular disease or diabetes type 2) involve body weight, BMI, lipids profile, blood pressure, plasma glucose and insulin levels. Nutrigenomics, a branch of nutritional and genomics science, which addresses the issue how diet influences genes, provides new developments in the field of nutrition and health that may help to develop new biomarkers (Palou 2007).

The field of nutrigenomics employs high-throughput OMICS technologies that enable to analyze the expression of large numbers of mRNAs (transcriptomics), proteins (proteomics), or metabolites (metabolomics) in a single experiment. The use of these platforms have the potential to deliver biomarkers, indicators of health and disease, assist in differentiating dietary responders from non-responders, as well as discover bioactive, beneficial food components (Kussmann et al. 2006). Transcriptomics generates comprehensive overview on molecular changes to dietary interventions in a given tissue at a given time, and it is suitable for a first screening in regulatory networks. The markers delivered by application of this approach may be useful to monitor the course of disease or the efficacy of treatment. Metabolomics is a diagnostic tool for metabolic classification of individuals using body fluids (urine, blood and saliva), and can deliver metabolic endpoints related to health or disease. In turn, proteomics is more complex in terms of absolute number, chemical properties and dynamic range of compounds present. It may deliver markers, as well as targets of intervention (enzymes or transporters) and is suitable for discovering bioactive food proteins and peptides (Kussmann et al. 2006; Kussmann et al. 2008). In the parallel to the biomarkers discovery, the use of transcriptomics may help to identify novel genes and pathways that could provide more information about disease or physiological changes occurring in the body (de Mello et al. 2012). Moreover, early transcript-based metabolic markers, which may emerge from this molecular approach, may be more beneficial than the conventionally used biomarkers, giving more time to intervene in the prevention of a disease that may develop in the future (de Mello et al. 2012).

On the other hand, studies on molecular mechanisms revealing effects of diet on health usually imply invasive tissue biopsies, such as subcutaneous adipose tissue (Viguerie et al. 2005). Suitability of biomarkers for efficient use requires that they can readily be assessed in

humans. Therefore, alternative, non-invasive ways to identify biomarkers to detect obesity and prevent its events are highly required.

3.2. Peripheral blood mononuclear cells (PBMCs) as a source of biomarkers

Blood is a complex tissue made up of plasma that transports a variety of blood cells – red blood cells (erythrocytes), white blood cells (leukocytes) and platelets – throughout the body. Red blood cells are the most abundant cells in the blood, mainly specialized in the transport of oxygen from the lungs to the tissues and return of carbon dioxide. These cells have no nucleus and it makes them more flexible when travelling through the blood vessels but also shortens their life-time at the same time. Unlike red blood cells, white blood cells are much fewer in number, contain single or multiple nuclei, and are mainly involved in defending the body against infections. The white blood cells can be divided into granulocytes (polymorphonuclear leukocytes): neutrophils, basophils, and eosinophils; and agranulocytes (mononuclear leukocytes): lymphocytes and monocytes. Lymphocytes contain a variety of cell types, including B-cells, T-cells and Natural killer (NK) cells. In turn, monocytes differentiate into macrophages in most tissues (Ziegler-Heitbrock, 2000). Peripheral blood mononuclear cells (PBMCs) are a subset of white blood cells including lymphocytes and monocytes. The relative proportion of each population of these cell types varies across individuals depending on health and disease states, as well as the response to different stimuli. In healthy adult individuals, the population of monocytes varies in the range 2-10% of PBMCs (Kasper D and Tinsley R 2005). In turn, within the lymphocyte subset, which are typically the largest cell population found in PBMCs, the relative proportion of T-cells varies in the range 61–85% and of B-lymphocytes in the range 7–23% (Reichert et al. 1991).

PBMCs have emerged as a convenient surrogate tissue for gene expression analysis in toxicological, nutritional and clinical studies, next to urine, sperm and hair follicles (Rockett et al. 2004). In humans, these blood cells can be easily and repeatedly collected in sufficient quantities in contrast to the target tissues, such as adipose, muscle and liver (de Mello et al. 2008a). Using peripheral blood cells as the source of gene expression material offers the possibility of sampling any individual at any time and also has the potential to detect early pathogenic and prognostic factors (Olsen et al. 2004). These cells travel through the body, and are able to respond to internal and external signals, thereby, can be used to assess biological responses as their gene expression profile may reflect the physiological and

pathological state of the organism (Burczynski and Dorner 2006; Bouwens et al. 2007; Manoel-Caetano et al. 2012).

PBMCs have been shown to express about 80% of the genes encoded by the human genome (Liew et al. 2006). Gene expression profiling of PBMCs has become increasingly explored recently in studies that address the issues related to health and disease. This approach has been used to develop prognostic and diagnostic biomarkers and to study mechanisms and phenotypes of various diseases, such as cancer (Showe et al. 2009), multiple sclerosis (Bomprezzi et al. 2003), ischemic stroke (Moore et al. 2005) and Down's syndrome (Tang et al. 2004). Surveys from these studies, usually employing microarray analysis, enabled to identify expression patterns of genes that may help to distinguish between control and disease groups. PBMCs have also been used as a model tissue in both human and animal studies addressing changes in gene expression levels in obesity and obesity-related diseases, such as hypertension and cardiovascular disease (Visvikis-Siest et al. 2007). In obese state, these cells have been found capable to express genes related to energy homeostasis, such as leptin (Samara et al. 2008), ghrelin (Mager et al. 2008), and could reflect the disturbance of insulin action (de Mello et al. 2008b). Moreover, PBMCs can also reflect the responses to dietary modifications (van Erk et al. 2006; Radler et al. 2011; Caimari et al. 2010b), fasting (Bouwens et al. 2007; Caimari et al. 2010a; Oliver et al. 2013), and to weight reduction strategies (Patalay et al. 2005; de Mello et al. 2008b). Interestingly, PBMCs seem to reflect liver environment (Boucher et al. 2000; Bouwens et al. 2007), as well as adipose tissue findings at gene expression levels (Caimari et al. 2010a). Therefore, it makes them a subject of great interest for transcriptomics profiling in dietary intervention studies, although, the application of microarrays in large and well-designed experiments would be required to explore and develop the potential gene expression signatures in the field of nutrigenomics (de Mello et al. 2012). All in all, in spite of raising number of studies that utilize PBMCs, the application of these cells in nutrigenomic research, compared to the use of central and peripheral tissues, is still less addressed.

II. OBJECTIVES AND EXPERIMENTAL DESIGN

OBJECTIVES AND EXPERIMENTAL DESIGN

One of the major challenges in developed societies is to implement therapeutic and, even more, preventive strategies, to struggle with global burden of obesity and its associated disorders. Specific dietary interventions and food components can contribute to this. Identification of efficient biomarkers is crucial to study the effectiveness of food-based strategies. However, the conventional biomarkers are those used in medicine and derive from disease processes, but there is lack of early biomarkers, especially accessible ones, that reflect physiological functionality before the phenotypic features become evident. In order to identify such biomarkers adequate experimental models are needed that result in early changes that reflect the later health status. Such models may be provided by maternal food restriction during pregnancy or during lactation, since have been previously described by our laboratory group to program different propensity to overweight/obesity and other alterations in adult life. Therefore, the principal aim of the present PhD thesis was **to identify early transcriptome-based biomarkers of metabolic functions with implications in health (metabolic health) and those related to body weight control and obesity, in peripheral blood mononuclear cells (PBMCs) of rats, which, as a result of interventions in the perinatal period, are more susceptible to obesity (model of maternal calorie restriction during pregnancy) or have developed certain protection against overweight and other associated alterations (model of maternal calorie restriction during lactation).** Furthermore, such experimental models can be used to ascertain which mechanisms of metabolic programming are involved, by analyzing morphological/histological changes and the expression of key genes in relevant tissues. The results of this project might serve in the future to implement personalized recommendations and nutritional interventions before the disease is manifested, as well as might serve as the basis for the design of new functional foods for the prevention of obesity.

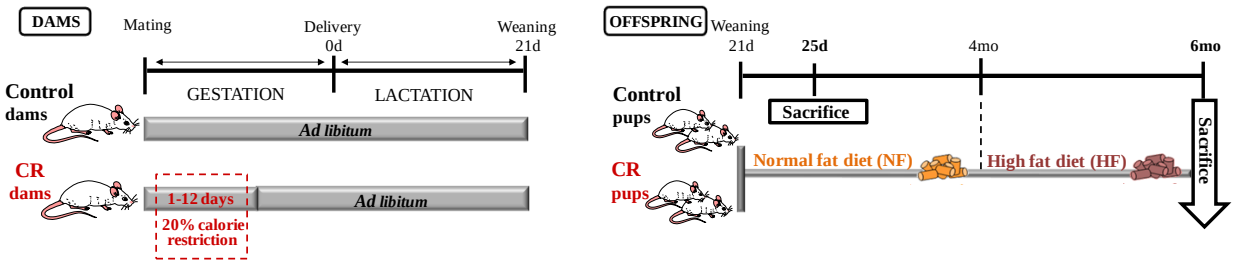
The research project was performed in the Laboratory of Molecular Biology, Nutrition and Biotechnology - Nutrigenomics (LBNB) - directed by Prof. Andreu Palou - at the University of the Balearic Islands (UIB) and CIBER de Fisiopatología de la Obesidad y Nutrición (CIBEROBN). In addition, in order to advance in the development of this research project and to broaden the PhD candidate formation, a training period of 3 months was realized in the laboratory of Prof. Jaap Keijer, Chair of Human and Animal Physiology at Wageningen University (The Netherlands). The purpose of this stay was to become familiar with the microarrays technique and to perform whole-genome transcriptome profiling of PBMCs

samples from the model moderate maternal calorie-restricted dams during lactation. The PhD project, including mentioned training stay abroad, was possible to be performed thanks to the scholarships awarded by Regional Government (CAIB) and European Social Fund. This project is a part of larger research projects founded by national and European bodies that are being developed in the LBNB, and more specifically of the European project "BIOmarkers of Robustness of Metabolic Homeostasis for Nutrigenomics-derived Health CLAIMS Made on Food (BIOCLAIMS)".

In order to achieve the principal aim of this PhD thesis four secondary objectives have been established and for each of them several specific tasks have been undertaken, as described in the next sections.

1. Identification of potential mechanisms by which moderate calorie restriction during pregnancy programs offspring for higher susceptibility to obesity in adulthood.

Moderate maternal calorie restriction (20%) during the first part of pregnancy in rats has been previously described by our laboratory group to program the offspring for greater propensity to develop obesity and related metabolic alterations in adulthood, particularly in male animals. Hence, the specific objective established was to gain insight into the possible programming mechanisms that may account for the adverse health outcomes in the offspring, by analyzing morphological and metabolic alterations in key organs involved in the control of energy homeostasis. For this reason, two groups of male and female rats were studied: the offspring of ad libitum fed dams (controls) and the offspring of 20% calorie restricted dams from day 1 to day 12 of pregnancy (CR).



Task 1.1. To analyse the effects of moderate maternal calorie restriction during pregnancy on determinants of later leptin and insulin resistance and to identify mechanisms responsible for the different outcomes in male and female animals. mRNA expression levels of genes involved in insulin and leptin signalling in hypothalamus, WAT and liver were analysed at a juvenile age (25 days) and in adulthood (6 months, after a 2-month period of HF diet

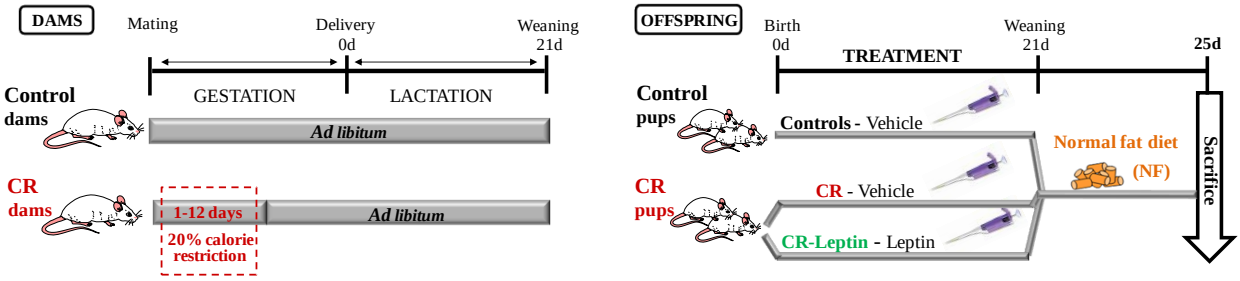
exposure), to determine whether this dietary stressor might set up early programmed disorders. The hormone leptin plays remarkable neurotrophic role in the development of hypothalamic circuitry, which coincides with naturally occurring surge of circulating leptin during short postnatal period. Thus, to ascertain whether the effects of 20% calorie restriction during gestation on later insulin and leptin homeostasis could be related with disturbances in leptin during the suckling period, circulating levels of leptin were determined in offspring at different stages of lactation. Results from this study are included in the **manuscript 1.**

Task 1.2. To assess whether the detrimental effects of undernutrition during gestation could be related to altered BAT thermogenic capacity and to investigate the potential mechanisms involved. Generation of heat in this fat tissue is mediated by UCP1 activation. In turn, activation of this protein is regulated by the SNS activity and secretions of hormones, including thyroid hormones. For this reason, in a first study, protein levels of UCP1 and of tyrosine hydroxylase (TyrOH), a marker of sympathetic innervation, as well as thyroid hormone signalling in BAT were analysed in young (25 days) control and CR rats belonging to the same cohort of animals described above (Task 1.1). Regarding the fact that BAT activity contributes to the maintenance of body temperature, the sensitivity of the offspring to cold was also determined in a second study. For this, a new cohort of control and CR animals was obtained and studied under cold conditions. Body temperature changes over time of 24-h cold-exposure and final BAT UCP1 levels were measured. Results obtained from these studies are described in the **manuscript 2.**

2. Testing the hypothesis that leptin supplementation during suckling period may revert malprogrammed effects of maternal calorie restriction during gestation.

It has been evidenced by our group and others that maternal calorie restriction during pregnancy brings about perturbations in postnatal leptin surge and hypothalamic circuitry that program impaired ability to regulate energy homeostasis in adulthood. Moreover, this prenatal condition has also been described to disturb sympathetic innervation of WAT, accompanied by adverse adiposity phenotype in adulthood. The hormone leptin is naturally present in significant amounts in breast milk and has been shown to play an essential and beneficial role during postnatal period. In this sense, previous studies in our laboratory group have shown that supplementation with physiological doses of leptin throughout lactation to neonate rats born to adequately nourished dams protect against the development of obesity and metabolic dysfunction in adulthood. Hence, we hypothesised that leptin supplementation

throughout lactation could be able to ameliorate malprogrammed sequels induced by poor prenatal nutrition. To complete this objective, three groups of male and female rats were studied at a juvenile age (25 days): the offspring of *ad libitum* fed dams (controls), the offspring of 20% calorie restricted dams during the first part of pregnancy (CR), and CR rats supplemented with physiological doses of leptin throughout lactation (CR-Leptin).



Task 2.1. To investigate the capacity of leptin supplementation throughout lactation to revert developmental malprogrammed effects exerted in offspring hypothalamus structure and function. For this purpose, morphometric analysis of two hypothalamic nuclei, ARC and PVN, as well as immunohistochemical studies on orexigenic neuropeptide Y in ARC and in PVN were performed in young (25 days) male and female animals belonging to the 3 groups described (controls, CR, CR-Leptin). In order to ascertain whether the treatments used influenced hypothalamic function, expression levels of genes encoding appetite-related neuropeptides and factors involved in leptin signalling were determined in hypothalamus of young offspring. Results from this study are described in the **manuscript 3.**

Task 2.2. To investigate whether leptin treatment during lactation is able to ameliorate the adverse programming effects of moderate maternal calorie restriction during gestation on WAT sympathetic innervation and function. Sympathetic innervation, by the analysis of TyrOH, both protein levels and its immunoreactive area, as well as thyroid hormone signalling, were determined in WAT of 25-day-old control, CR, and CR-Leptin animals. In addition, since it is well established that SNS and thyroid hormones play a critical role in adipocyte lipid metabolism, mRNA expression levels of genes involved in lipolysis and fatty acid oxidation, as well as in fuel supply into tissue were also explored. Results obtained from this study are described in the **manuscript 4.**

3. Identification of early transcriptome-based biomarkers, as potential indicators of both programmed susceptibility to later disorders due to maternal calorie restriction during pregnancy and response to neonatal leptin treatment during the suckling period

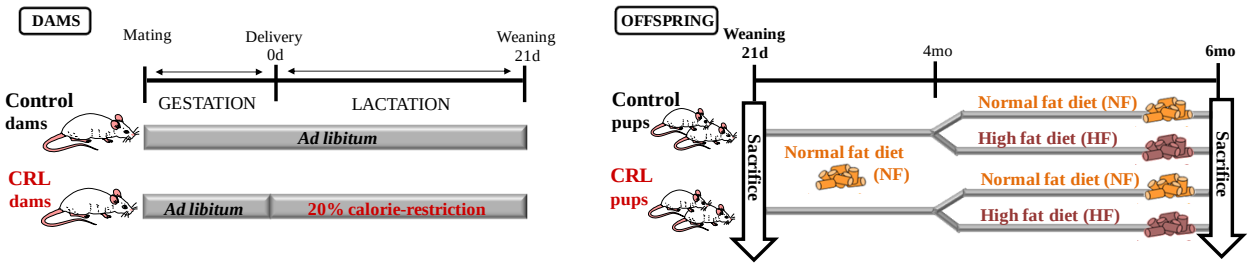
The mechanisms contributing to obesity and its related chronic pathologies are complex. Hence, the discovery of novel multiple biomarkers that reflect a wide system of biological processes, beyond the traditional risk factors for obesity development, may improve the accuracy of its detection. Moreover, identification of biomarkers that predict the propensity to these alterations before the disease is manifested, and especially those that are easily accessible, may give more time to prevent future disease and improve monitoring of the efficacy of food-based strategies used. Considering that calorie restriction during gestation increases the propensity to develop obesity and other alterations in later life, whereas leptin treatment during the suckling period reverts, at least in part, these malprogramming effects, we undertook these issues to identify transcript-based early biomarkers in PBMCs, an easily accessible surrogate tissue, that may predict programmed susceptibility to chronic diseases and to monitor its potential reversion.

Task 3.1. Whole genome profiling of PBMCs obtained from 25 day-old animals belonging to the same cohort of animals described in objective 2 (control, CR and CR-Leptin) was performed using microarray technology. Gene array findings were validated by analysing expression levels of a selection of genes in PBMC samples by RT-qPCR. Results obtained from this study have been included in a patent application and are described in the **manuscript 5.**

4. Characterization of the offspring of rats submitted to the moderate calorie restriction during lactation and use of this animal model to identify early potential transcriptomic-based biomarkers of improved metabolic health

Unlike maternal undernutrition during pregnancy, 30% maternal calorie restriction in lactating dams has been previously described to protect the offspring against development of diet-induced obesity and its related metabolic alterations in adulthood. The beneficial effects of this condition were particularly manifested by improved capacity to handle and store excess dietary fuel. The present study was conducted to determine whether a less severe calorie restriction of 20%, which could be more easily representative or applicable in humans, could also confer beneficial effects in the offspring. If so, this model could also be

useful to identify early transcriptomic-based biomarkers of improved metabolic health. For this, male and female offspring of control and 20% calorie-restricted dams during lactation (CRL) were studied.



Task 4.1. To characterize metabolic health of the offspring of 20% calorie restricted dams during lactation by analyzing phenotypic traits and circulating parameters at early ages and in adulthood. Changes occurring at the transcriptional level in genes related to lipid metabolism and insulin and leptin signaling in WAT and liver were studied, both at early stages and in adulthood (6 months) under normal-fat and high-fat diet conditions. The potential relation between gene expression patterns and the improvement of metabolic health was also evaluated. Results from this study are described in the **manuscript 6**.

Task 4.2. To use the model of moderate maternal calorie restriction of 20% during lactation to identify early potentially predictive biomarkers of metabolic health in relation to lipid metabolism processes by transcriptome profiling of PBMC samples. Whole-genome microarray analysis of PBMCs from 21-day-old offspring of control and 20% calorie-restricted dams during lactation was performed. Transcripts of selected genes related to lipid metabolism identified at early ages were analysed in adult rats (6 months), to check the variability over time of potential markers. Moreover, the expression pattern of these genes were also assessed in metabolically relevant tissues, such as liver and WAT, at both time points. Results from this study are collected in the **manuscript 7.**

III. RESULTS AND DISCUSSION

MANUSCRIPT 1

Impaired insulin and leptin sensitivity in the offspring of moderate caloric restricted dams during gestation is early programmed

Palou, M., Konieczna, J., Torrens, J.M., Sánchez, J., Priego, T., Fernandes, M.L., Palou, A., and Pico, C.

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Title page

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Conflict of interest: None

Abstract

We aimed to assess the mechanisms responsible for hyperphagia and metabolic alterations caused by maternal moderate caloric restriction during gestation. Male and female offspring of control and 20% caloric-restricted rats (CR) were studied. They were fed a normal-fat diet until 4-months of age, and then moved to a high-fat diet until 6-months of age. Blood parameters and expression of selected genes in hypothalamus, retroperitoneal white adipose tissue (rWAT) and liver were analyzed at 25-days and 6-months of age. Plasma leptin was measured during suckling. Levels of proteins involved in insulin and leptin signaling were determined at 6-months of age. CR ate more calories than controls, but only males gained more weight. A peak in plasma leptin was found in 9-day-old controls, but was absent in CR. 25-day-old CR showed lower Insr mRNA levels in hypothalamus, rWAT and liver, and of Obrb in hypothalamus. At the age of 6-months, HOMA-IR index was higher in CR than controls, and CR males also displayed hyperleptinemia. Adult CR also showed lower Obrb mRNA levels in the hypothalamus (only females, but both showed altered Npy/Pomc mRNA ratio), rWAT, and liver (males), and a decrease in PKCζ levels, in rWAT (females) and liver (males), and of pSTAT3 in liver (females). These results suggest CR animals are programmed for insulin and central leptin resistance, which may explain the dysregulation of appetite and other metabolic alterations, favoring obesity development, although only manifested in males. These early programming effects could be associated with the absence of leptin surge during lactation.

Key words: insulin and leptin sensitivity, caloric restriction, gestation, early programming, leptin surge

Introduction

It is becoming increasingly clear that environmental conditions during critical periods of development may lead to differential programming of the mechanisms involved in the control of energy balance (McMillen et al. 2008). Gestation and lactation are considered critical periods for development, and food restriction during these periods has been described to induce permanent adaptive changes that may have lasting effects on the metabolic regulatory mechanisms of the offspring, leading to different outcomes in the propensity to suffer obesity in adult life (Martin-Gronert and Ozanne 2006; McMillen et al. 2005). While moderate caloric restriction during lactation has been associated with certain protection against later obesity in rats (Palou et al. 2010b; Palou et al. 2011b), caloric restriction during pregnancy has been reported to be a risk factor increasing the vulnerability to later obesogenic environmental stimuli (McMillen et al. 2008; Thompson et al. 2007; Palou et al. 2010a), in both cases with different outputs depending on the severity or type of restriction and also on the gender of animals (Palou et al. 2010b; Palou et al. 2011b; Palou et al. 2010a). In this sense, we have previously described that 20% caloric restriction in rats during the first half of gestation results in higher food intake in their offspring in adulthood and this concludes in higher body weight in males but not in females (Palou et al. 2010a).

Central resistance to insulin and/or leptin has been proposed as important mechanisms responsible for the dysregulation of energy homeostasis, which may lead to obesity (Palou et al. 2010a; Levin and Dunn-Meynell 2002; Lustig et al. 2004). In fact, the offspring of 20% caloric restricted animals during gestation, both males and females, display hyperinsulinemia, which is already present at a juvenile age, and previous to any apparent effect on body weight (Palou et al. 2010a). In addition, these animals, but only males, also display hyperleptinemia in adulthood, when exposed to a HF diet (Palou et al. 2010a). These results suggest that mechanisms involved in insulin and/or leptin sensibility could have been affected as a consequence of this prenatal condition, with these later consequences on body weight control capacity. However, the concrete mechanisms involved and why programming mechanisms had different outcomes in male and female were not determined.

The hypothalamus is the main organ responsible for the central control of energy balance and appetite behavior, by the production of many neuropeptides and the

establishment of sympathetic connections, responding to different stimuli, such as the circulating hormones insulin and leptin (Schwartz and Porte, Jr. 2005; Schwartz et al. 2000); the brain is particularly sensitive during the perinatal period and it has been described to undergo alterations in response to particular nutritional conditions during fetal development and neonatal life (McMillen et al. 2005). Leptin has been shown to play an important role during the perinatal period (Palou and Pico 2009). In fact, supplementation to neonate rats with physiological doses of leptin during lactation has been described to improve body weight control and leptin and insulin sensitivity in adult life (Pico et al. 2007; Sanchez et al. 2008), while a lack of leptin during this period occurring in leptin-deficient mice disrupts the normal postnatal developmental pattern of neural projection in the hypothalamus (Bouret et al. 2004). Previous studies have shown that maternal 20% caloric restriction during the first half of pregnancy resulted in lower cellularity and neuropeptide Y (NPY) - and α-melanocyte-stimulating hormone (αMSH)-neurons in the arcuate nucleus (ACR) in the offspring (Garcia et al. 2010). Delahaye *et al.* (Delahaye et al. 2008) also described that maternal severe (50%) caloric restriction during both gestation and lactation reduced fiber projections from ARC neurons to other hypothalamic structures and decreased proopiomelanocortin (Pomc) and αMsh mRNA expression levels in neonate rats. In addition, adult offspring from 30% caloric restricted pregnant dams have been shown to have the hypothalamic gene expression of Pomc, Npy, Agouti-related protein (Agrp) and leptin receptor (Obrb) altered (Ikenasio-Thorpe et al. 2007). In addition, without neglecting the important role of the hypothalamus in regulating energy homeostasis, both white adipose tissue (WAT) and liver are also key organs in the regulation of energy balance and substrate metabolism and targets of the peripheral actions of insulin and leptin (Palou et al. 2008). In fact, the response of these tissues to feeding conditions may be another major factor determining the higher susceptibility to developing obesity and related metabolic alterations (Palou et al. 2008; Priego et al. 2008).

Thus the aim of the present study was to determine the effects of moderate (20%) maternal caloric restriction during the first 12 days of gestation on determinants of later leptin and insulin resistance, by exploring the expression of selected genes involved in insulin and leptin signaling in key tissues such as the hypothalamus, the retroperitoneal WAT (rWAT) and the liver, both at a juvenile age (25 days) and in adulthood (6 months, after a 2-month-period of HF diet exposure), to analyze whether this dietary

stressor is able to step up early programmed disorders. Ultimately, considering the important role of leptin during a critical window of development, it was also the aim of this study to ascertain whether the effects of caloric restriction during gestation on later leptin and insulin homeostasis could be related, in part, with an alteration or a deficiency in leptin during the suckling period.

Materials and Methods

Animals and experimental design

The study was performed in male and female rats from 32 different litters, following the protocol below. All rats were housed under controlled temperature (22 ºC) and a 12 h light–dark cycle (light on from 08:00 to 20:00), and had unlimited access to tap water and standard chow diet (3 kcal/g, with 2.9% calories from fat; Panlab, Barcelona, Spain) unless mentioned otherwise. Briefly, virgin female Wistar rats weighing between 200 g and 225 g were mated with male rats (Charles River Laboratories, Barcelona, Spain). Day of conception (day 0 of pregnancy) was determined by examination of vaginal smears for the presence of sperm, and then female rats were single caged. Pregnant rats were divided into two groups: one with free access to standard chow diet, and the other one underwent 20% restriction of caloric intake from day 1 to day 12 of pregnancy. Caloric restriction was performed by offering each dam a daily amount of food corresponding to 80% of the calories that should be eaten according to body weight. This amount was calculated considering the calories daily consumed by their control animals under *ad libitum* feeding conditions. After the caloric restriction period, rats were allowed to eat *ad libitum*, and food intake was measured. At day 1 after delivery, excess pups of each sex in each litter were removed to keep 10 pups per dam (five males and five females, when possible). Weaning was conducted at 21 days of life.

One set of animals from 10 control dams and from 9 caloric restricted dams was used to obtain blood samples at different stages of lactation (days 5, 9 and 15 of lactation), under *ad libitum* feeding conditions (n= 5-10 animals/group). Blood was collected in heparinized containers to obtain plasma for leptin determination.

On day 25 of life, another set of control and CR animals (n= 5-7 animals/group) (from 6 and 8 dams, respectively) were killed by decapitation under fed conditions, during the first 2 h at the beginning of the light cycle. Blood samples were collected in heparinized

containers, then centrifuged at 700 g for 10 min to obtain the plasma, and stored at -20ºC until analysis. The hypothalamus, rWAT depot and the liver were rapidly removed, weighed, frozen in liquid nitrogen and stored at -80ºC until ulterior studies.

At weaning, a third set of animals from the same dams as those killed on day 25 of life, including 24 controls (12 males and 12 females) and 28 CR animals (12 males and 16 females), was kept alive.. They were placed two per cage, paired with another animal of the same group, and fed with standard diet until the age of 4 months; then they were exposed to a high fat (HF) diet (4.7 kcal/g, with 45% calories from fat, Research Diets, Inc., NJ, USA) until the age of 6 months. HF diet contained 5.5% calories from soybean oil and 39.5% from lard. Body weight and food intake of the offspring were followed.

At the age of 6 months, both control and CR rats were killed under two feeding conditions: *ad libitum* feeding conditions (fed group, n=6-8/group), and 12-h fasting conditions (fasted group, n=6-8/group). All animals were sacrificed by decapitation during the first 2 h of the beginning at the light cycle and on different consecutive days (including animals from each group every day). Blood samples were collected in heparinized containers, then centrifuged at 700 g for 10 min to obtain the plasma, and stored at -20ºC until analysis. The hypothalamus, WAT depots (retroperitoneal, mesenteric, gonadal and inguinal) and liver were rapidly removed, weighed, frozen in liquid nitrogen and stored at -80ºC until ulterior studies.

Although different WAT depots were sampled to be weighed, the retroperitoneal depot was selected as representative to be analyzed for gene expression, based in literature showing that this depot seems to be more sensitive to nutritional status, compared with other depots (Palou et al. 2010c).

The animal protocol followed in this study was reviewed and approved by the Bioethical Committee of our University and guidelines for the use and care of laboratory animals of the University were followed.

Measurement of circulating parameters under fed/fasting conditions, and calculation of the homeostatic model assessment for insulin resistance (under fasting conditions)

Blood glucose concentration was measured using Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Plasma insulin concentration was determined using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia AB, Uppsala,

Sweden) following standard procedures. Plasma leptin concentration was measured using a mouse leptin ELISA kit (R&D Systems, Minneapolis, MN). Circulating triglycerides (TG) were measured by commercial enzymatic colorimetric kit (Triglyceride (INT), Sigma Diagnostics, St Louis, MO, USA).

The homeostatic model assessment for insulin resistance (HOMA-IR) was used to assess insulin resistance. It was calculated from fasting insulin and glucose concentration using the formula of Matthews *et al.* (Matthews et al. 1985): HOMA-IR = fasting glucose (mmol/liter) x fasting insulin (mU/liter)/22.5.

RNA extraction

Total RNA was extracted from the hypothalamus, rWAT and the liver by Tripure Reagent (Roche Diagnostic Gmbh, Mannheim, Germany) according to the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies Inc., Wilmington, Delaware, USA) and its integrity confirmed using agarose gel electrophoresis.

Real-time quantitative PCR (RT-qPCR) analysis

Real-time polymerase chain reaction (PCR) was used to measure mRNA expression levels of long form leptin receptor (Obrb) and insulin receptor (Insr) in hypothalamus, rWAT and liver of 25-day and 6 month-old rats; neuropeptide Y (Npy) and proopiomelanocortin (Pomc) in hypothalamus, tumor necrosis factor alpha (Tnfα), adipose triglyceride lipase (Atgl) and carnitine palmitoyltransferase 1 (Cpt1) in rWAT, and sterol response element binding protein 1c (Srebp1c), acetyl-coenzyme A carboxylase alpha (Acc1) and glycerol-3-phosphate acyltransferase (Gpat) in liver, in rats of 6 months of age. 0.25 µg of total RNA (in a final volume of 5 µl) was denatured at 65 ºC for 10 min and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20ºC for 15 min, 42ºC for 30 min, with a final step of 5 min at 95ºC in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain). Each PCR was performed from diluted cDNA template, forward and reverse primers (1 µM each), and Power SYBER Green PCR Master Mix (Applied Biosystems, CA, USA). Primers were obtained from Sigma (Madrid, Spain) and sequences are described in Table 1. Real time PCR was performed using the Applied Biosystems StepOnePlusTM Real-Time PCR Systems (Applied

Biosystems) with the following profile: 10 min at 95ºC, followed by a total of 40 two-temperature cycles (15 s at 95ºC and 1 min at 60ºC). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.0) and the relative expression of each mRNA was calculated as a percentage of NF control rats under *ad libitum* feeding conditions, using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). Beta-actin and Gdi1 were used as reference genes depending on the tissue, age and sex of the animals, according to their better suitability.

Table 1. Nucleotide sequences of primers used for PCR amplification.

Gene	Fordward Primer (5' to 3')	Reverse Primer (5' to 3')	Amplicon size (pb)
β-actin	GAAGCTGTGCTATGTTGCCC	GGATTCCATACCCAGGAAGG	184
Gdi1	CCGCACAAGGCAAATACATC	GACTCTCTGAACCGTCATCAA	210
Insr	GTCCGGCGTTCATCAGAG	CTCCTGGGATTCATGCTGTT	242
Obrb	AGCCAAACAAAAGCACCATT	TCCTGAGCCATCCAGTCTCT	174
Leptin	TTCACACACGCAGTCGGTAT	AGGTCTCGCAGGTTCTCCAG	186
Npy	TGGACTGACCCTCGCTCTAT	GTGTCTCAGGGCTGGATCTC	188
Pomc	CCTGTGAAGGTGTACCCCAATGTC	CACGTTCTTGATGATGGCGTTC	266
Tnfα	CCGATTTGCCATTTCATACC	TCGCTTCACAGAGCAATGAC	230
Atgl	TGTGGCCTCATTCCTCCTAC	AGCCCTGTTTGCACATCTCT	271
Cpt1	GCAAACTGGACCGAGAAGAG	CCTTGAAGAAGCGACCTTTG	180
Srebp1c	AGCCATGGATTGCACATTTG	GGTACATCTTTACAGCAGTG	260
Acc1	TGCAGGTATCCCCACTCTTC	TTCTGATTCCCTTCCCTCCT	212
Gpat	CAGCGTGATTGCTACCTGAA	CTCTCCGTCCTGGTGAGAAG	194

Abbreviations: Gdi1, guanosine diphosphate dissociation inhibitor 1; Insr, insulin receptor; Obrb, long-form leptin receptor; Npy, neuropeptide Y; Pomc, proopiomelanocortin; Tnfα, tumor necrosis factor alpha; Atgl, adipose triglyceride lipase; Cpt1, carnitine palmitoyltransferase 1; Srebp1c, sterol response element binding protein 1c; Acc1, acetyl-coenzyme A carboxylase alpha; Gpat, glycerol-3-phosphate acyltransferase.

Western blot analysis

The amount of total insulin receptor substrate 1 (IRS1), phosphorilated IRS1 on Try612 (pIRS1), protein kinase C zeta (PKCζ), signal transducer and activator of transcription 3 (STAT3) and tyrosine 705 phosphorylated STAT3 (pSTAT3) in rWAT and liver of control and CR rats at the age of 6 months were determined by Western blot. Tissue was homogenized at 4 ºC in 1:3 (w:v) or 1:20 (w:v), for rWAT and liver respectively, of lysis buffer as previously described in (Mercader et al. 2006). The homogenate was centrifuged at 500 g for 10 min at 4 ºC and the supernatant was used for protein analysis. Total protein content was measured by the method of Bradford (Bradford 1976). For analysis, 300 μg (for rWAT analysis) or 30μg (for liver analysis) of total protein was solubilized and boiled for 3 min in Laemmli sample buffer containing 5% 2-beta-mercaptoethanol. Then, total protein was fractionated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE; 7.5 % polyacrylamide) and electrotransferred onto a nitrocellulose membrane (Bio-Rad, Madrid, Spain). Black amide B10 staining provided visual evidence for correct loading and blotting of proteins. After blocking, the membrane was incubated with the primary rabbit polyclonal anti-IRS1, anti-pIRS1(Tyr632), anti-PKCζ, anti-STAT3 or anti-pSTAT3(Tyr705) antibody (Santa Cruz Biotechnology, Inc., CA, USA), and then with the infrared (IR)-dyed secondary anti-IgG antibody (LI-COR Biociences, Nebraska, USA) diluted 1:10000. For IR detection membranes were scanned in Odyssed Infrared Imaging System (LI-COR Biociences, Nebraska, USA), and the bands were quantified using the analysis software provided.

Statistical analysis

Data were expressed as mean ± S.E.M. Multiple comparisons were assessed by one-, two- and three-way ANOVA to determine the effects of different factors (sex, caloric restriction during pregnancy, feeding conditions and/or the day of lactation). Single comparisons between groups were assessed by Student's t test. The analyses were performed with SPSS for Windows (SPSS, Chicago, IL). $P<0.05$ was the threshold of significance.

Results

Body weight gain and cumulative food intake of control and CR animals until the age of 6 months

Moderate caloric restriction during the first 12 days of gestation resulted in higher body weight in the male offspring from day 74 of life onwards (Student's t test) compared with their controls (Figure 1A). When animals were 4 months old (just before changing to HF diet), CR male animals weighed 6.9% more than their controls, and the difference was even higher (12.5%) when animals were 6 months old and were under HF diet. Unlike males, no significant changes concerning body weight were found between control and CR female animals, either under NF diet or HF diet.

As shown in Figure 1B, cumulative food intake from weaning until the age of 6 months was significantly higher in both male and female CR compared with their controls. These differences were found during the feeding period with the NF diet, as well as, and even higher, when they were under HF diet.

Circulating leptin levels in control and CR pups during lactation

No significant differences were found in body weight between control and CR animals at birth, or during lactation (data not shown).

Circulating leptin levels were studied at different stages of lactation (5, 9 and 15 days of life) (Figure 2). No significant differences were found between control and CR animals concerning leptin levels on days 5, 9 and 15 of lactation. However, interestingly, male and female control animals showed a surge of circulating leptin concentration at the age of 9 days (one-way ANOVA), in contrast with CR rats which maintained similar levels in the 3 days of lactation analyzed (interaction between the effect of caloric restriction and the day of lactation, three-way ANOVA).

Body weight, tissue weights, and blood parameters in control and CR rats at the age of 25 days

As previously described in the same cohort of animals, no significant differences were found in body weight between control and CR animals at the age of 25 days (two-way ANOVA) (Garcia et al. 2010) (Table 2). No significant differences were found in the weight of the retroperitoneal WAT and liver either (Table 2).

Concerning blood parameters, blood glucose and plasma insulin levels were not significantly different in CR animals compared with their controls (two-way ANOVA). Circulating leptin concentration was lower in CR male rats, but not in females, with respect to their controls (Student's t test) (Table 2).

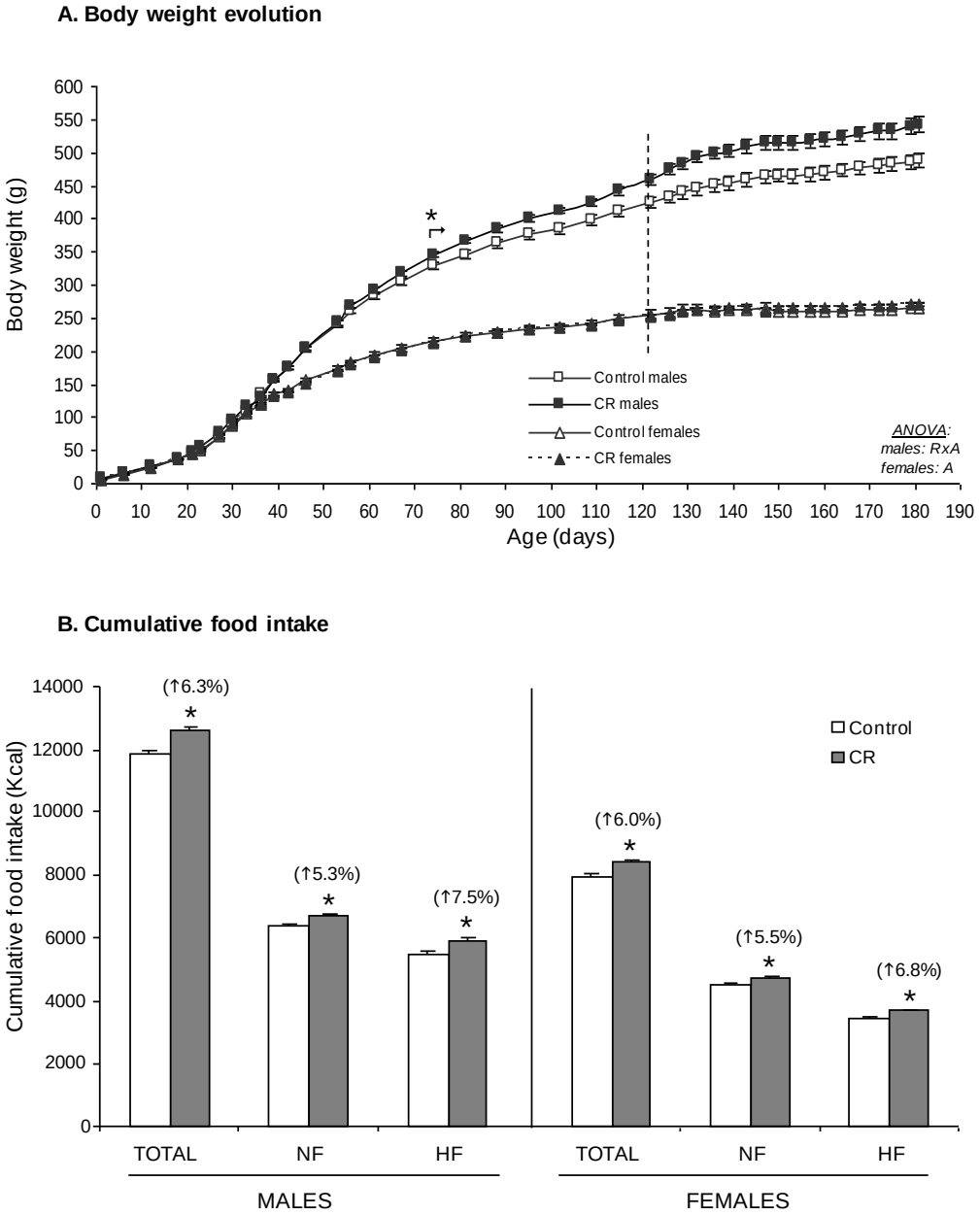

Figure 1. (A) Body weight over time until the age of 6 months of male and female offspring of controls and caloric restricted dams during gestation (CR). Animals were fed with standard normal fat (NF) diet until the age of 4 months and then exposed to a high fat (HF) diet (the dotted line indicates the time point of change from NF to HF diet). (B) Cumulative caloric intake from weaning at the age of 21 days until 6 months of age (TOTAL), as well as during NF diet feeding (from 21 days to 4 months old) (NF) and when animals were exposed to HF diet (from 4 to 6 months old) (HF) of male and female control and CR rats. The percentage increase in food intake of CR compared with controls is indicated in brackets. Data are expressed as the mean ± SEM of 12-16 animals per group. Statistics: *A*, effect of age; *RxA*, interaction between caloric restriction during gestation and age ($p<0.05$, ANOVA repeated measures). *, different from their respective control group ($p<0.05$, Student's t test). The arrow indicates the starting point of significant effects on body weight in male animals.

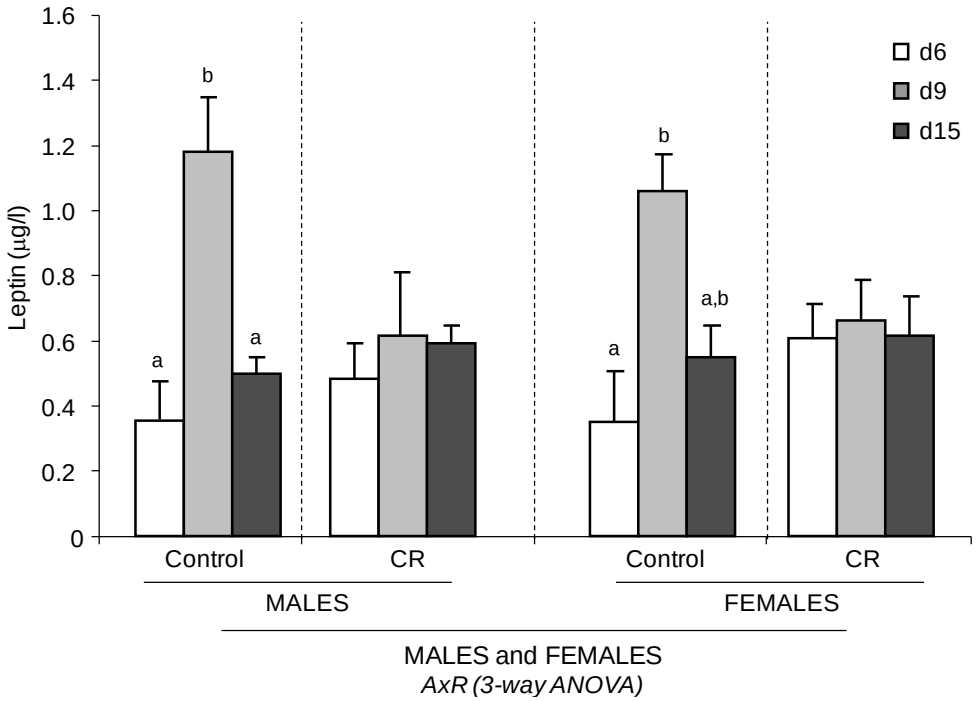

Figure 2. Plasma leptin levels (µg/l) of male and female offspring of controls and caloric restricted dams during gestation (CR) at different stages of lactation: 6 days (d6), 9 days (d9) and 15 days (d15) of life. Data are means ± SEM (n = 5 to 10). Statistics: RxA, interaction between caloric restriction (R) and the day of lactation (A) in male and female pups ($p<0.05$, three-way ANOVA). Within each graph, bars not sharing a common letter (a, b) are significantly different ($p<0.05$, one-way ANOVA and Bonferroni post-hoc test).

Table 2. Weight-related parameter and blood parameters at the age of 25 days.

	Males		Females	
	Control	CR	Control	CR
Body weight (g)	61.4 ± 2.8	59.3 ± 2.0	58.6 ± 2.3	55.7 ± 2.8
Liver weight (mg)	2.86 ± 0.11	2.60 ± 0.11	2.79 ± 0.07	2.69 ± 0.12
rWAT weight (g)	113 ± 11	95.7 ± 7.8	95.4 ± 16.5	92.2 ± 18.4
Glucose (mg/dl)	130 ± 6	137 ± 3	124 ± 4	131 ± 6
Insulin (ng/l)	170 ± 38	122 ± 36	154 ± 41	167 ± 35
Leptin (ng/l)	959 ± 119	608 ± 51 *	984 ± 164	841 ± 40

Body weight, liver and rWAT weight, and circulating glucose, insulin and leptin levels at 25 days of life (n=6–8) of male and female offspring from controls and caloric restricted dams during gestation (CR) under *ad libitum* feeding conditions. Data are mean±S.E.M. Statistics: No significant differences were found by two-way ANOVA; *, different from their respective control group ($p<0.05$, Student's t test).

Gene expression levels in hypothalamus, retroperitoneal WAT and liver

Figure 3 shows mRNA expression levels of Insr and Obrb in the hypothalamus, rWAT and liver of control and CR animals at the age of 25 days. Interestingly, both male and

female CR animals displayed lower Insr and Obrb mRNA expression levels in the hypothalamus compared with their controls (two-way ANOVA). In rWAT and liver, Insr mRNA levels were also significantly lower in CR animals compared with their controls (two-way ANOVA), but no significant differences were found concerning Obrb mRNA levels.

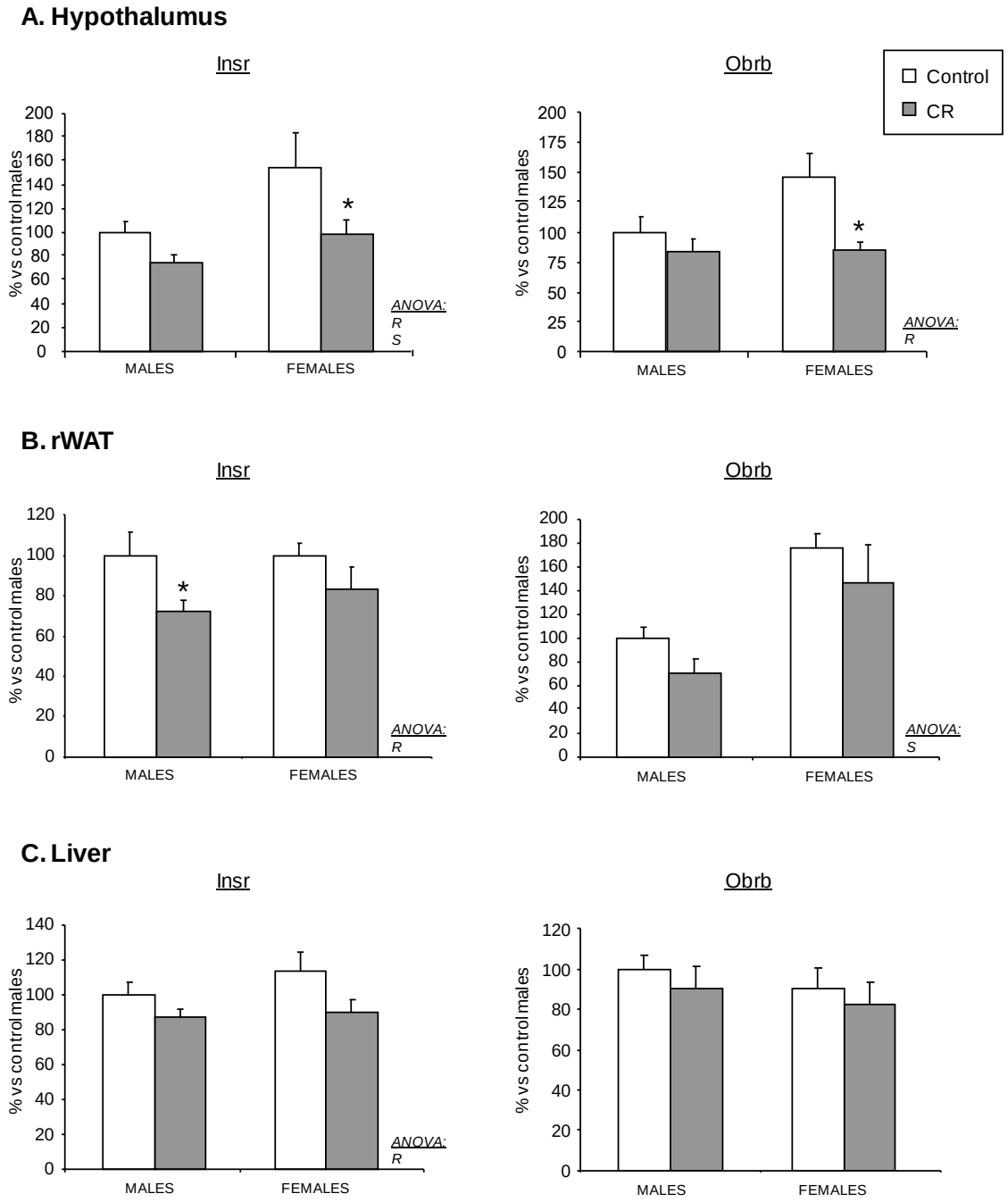

Figure 3. Insr and Obrb mRNA expression levels in hypothalamus (A), rWAT (B) and liver (C) of 25-day-old male and female offspring of controls and caloric restricted dams during gestation (CR). mRNA levels were measured by Real-time PCR and expressed as a percentage of the value of control male rats. Data are mean ± S.E.M. (n=6-8). Statistics: R, effect of caloric restriction, and S, effect of sex, ($p<0.05$, two-way ANOVA). *, different from their respective control group ($p<0.05$; Student's t test).

Body weight, tissue weights and blood parameters under fed and fasting conditions in control and CR rats at the age of 6 months

As described above, moderate maternal caloric restriction during gestation resulted in higher body weight in adulthood of male offspring but not of females (two-way ANOVA). 12-h fasting induced a significant decrease of body weight in all animals (ANOVA repeated measures) (Table 3).

The differences in body weight between control and CR male rats can be attributed to the size of fat depots (Table 3). The adiposity index was higher in CR males with respect to their controls (two-way ANOVA). In fact, CR male animals showed greater fat pad weights in the 4 depots weighed, in comparison to controls (two-way ANOVA).

Although no significant differences were found between control and CR female animals, a tendency to higher adiposity was found in the latter ($p=0.066$, two-way ANOVA) especially due to the significantly greater gonadal fat depot (two-way ANOVA). In both males and females, no significant differences were observed by the effect of 12-h fasting in the weights of the fat depots studied, and even an increase in the size of rWAT was observed in the group of female control animals after 12 h fasting. This difference does not seem to be a direct effect of fasting, but may probably be attributed to differences in the initial weight of fat depots between animals, which could also be masking other significant effects due to the fasting state. On the other hand, gestational caloric restriction resulted in higher liver weight in CR male animals but not in females (two-way ANOVA), especially under fasting conditions (Student's t test). As expected, fasted rats from the different groups showed lower liver weight than fed rats (two-way ANOVA).

Table 4 shows circulating glucose, insulin, leptin, and TG levels of male and female control and CR animals under feeding conditions and after 12-h fasting. No significant differences were found in glucose levels between control and CR rats (two-way ANOVA). Fasted rats presented lower glucose levels than fed animals (two-way ANOVA). Insulin levels also decreased after fasting in male and female control and CR animals (two-way ANOVA), although a little non significant response by Student's t test was found in CR male animals after food deprivation. In fact, CR male rats showed higher insulin concentration under fasting conditions in comparison to their controls (Student's t test). In females, no significant differences were observed between control

and CR rats concerning circulating insulin levels (Student's t test).

HOMA-IR was calculated to estimate insulin resistance (Figure 4). Notably, both male and female CR animals showed a higher HOMA-IR index compared with their controls (two-way ANOVA), although the increase was more pronounced and only significant by Student's t test in CR male rats, which showed 177% increase *vs* controls, in contrast with the 31% increase found in CR females. It should also be mentioned that HOMA-IR value was significantly lower in females compared with males (two-way ANOVA).

Table 3. Weight-related parameters at the age of 6 months.

	Control		CR		
	Ad Libitum	12-h fasting	*Ad Libitum*	12-h fasting	*ANOVA*
Males					
Body weight before fasting (g)		495 ± 20		546 ± 15	
Body weight at sacrifice (g)	487 ± 9	486 ± 20	548 ± 20 *	536 ± 15	*R*
Liver (g)	14.2 ± 0.4	10.2 ± 0.6 #	15.4 ± 0.4	11.9 ± 0.5 * #	*R,F*
rWAT (g)	15.4 ± 1.1	13.6 ± 1.7	19.0 ± 1.7 *	17.6 ± 1.7	*R*
mWAT(g)	6.53 ± 0.73	5.79 ± 0.99	9.56 ± 1.06 *	8.04 ± 1.04	*R*
iWAT (g)	11.7 ± 1.1	11.3 ± 2.0	16.3 ± 1.6 *	15.1 ± 0.8	*R*
gWAT (g)	16.0 ± 1.3	15.1 ± 1.7	22.9 ± 1.7 *	20.8 ± 1.5 *	*R*
Adiposity index (%)	10.1 ± 0.6	9.32 ± 0.81	12.3 ± 0.6	11.4 ± 0.6	*R*
Females					
Body weight before fasting (g)		269 ± 7		269 ± 4	
Body weight at sacrifice (g)	263 ± 14	261 ± 7	274 ± 5	262 ± 5	
Liver (g)	7.5 ± 0.4	6.1 ± 0.3 #	7.8 ± 0.3	6.1 ± 0.2 #	*F*
rWAT (g)	2.96 ± 0.34	4.13 ± 0.39 #	4.43 ± 0.61	3.89 ± 0.42	
mWAT(g)	2.45 ± 0.34	2.10 ± 0.17	2.83 ± 0.27	2.53 ± 0.39	
iWAT (g)	3.54 ± 0.49	3.10 ± 0.29	3.83 ± 0.38	3.84 ± 0.35	
gWAT (g)	7.64 ± 1.21	8.62 ± 0.73	9.79 ± 1.01	10.8 ± 1.0	*R*
Adiposity index (%)	6.18 ± 0.59	6.88 ± 0.81	7.59 ± 0.52	7.94 ± 0.64	

Weight-related parameters at the age of 6 months from male and female offspring of controls and caloric restricted dams during gestation (CR), under *ad libitum* feeding conditions and after 12 h fasting conditions. Data are mean±S.E.M. (n=6-8). Statistics: R, effect of caloric restriction, and F effect of fasting (two-way ANOVA); *, different from their respective control group, and #, different from fed conditions (Student's t test). 12-h fasting induced a significant decrease of body weight in all animals ($p<0.05$, effect of fasting and sex, ANOVA repeated measures).

Regarding leptin (Table 4), CR male animals displayed higher circulating leptin levels than controls (two-way ANOVA), especially under fasting conditions (Student's t test).

In contrast, no differences were found between control and CR females (two-way ANOVA). As expected, circulating leptin levels decreased after 12-h fasting in male and female control and CR rats (two-way ANOVA).

Concerning TG, no differences were observed as a consequence of the caloric restriction or fasting conditions in both male and female animals (two-way ANOVA). However, it is interesting to highlight that control male rats showed a significant decrease in circulating TG levels after fasting (Student's t test) which was not present in CR males.

Table 4. Circulating parameters at the age of 6 months.

	Control		CR		
	Ad Libitum	12-h fasting	*Ad Libitum*	12-h fasting	*ANOVA*
Males					
Glucose (mg/dl)	110 ± 2	89.3 ± 5.9 #	113 ± 5	95.8 ± 2.3 #	*F*
Insulin (µg/l)	2.52 ± 0.42	0.510 ± 0.069 #	2.11 ± 0.35	1.43 ± 0.33 *	*F*
Leptin (µg/l)	11.6 ± 1.7	3.48 ± 0.48 #	14.8 ± 2.3	7.05 ± 0.89 * #	*R,F*
Triglycerides (g/l)	3.93 ± 0.18	2.44± 0.29 #	4.38 ± 0.85	5.32 ± 1.89	
NEFA (mmol/l)	0.959 ± 0.053	0.727± 0.042 #	1.08 ± 0.12	0.751 ± 0.060 #	*F*
Females					
Glucose (mg/dl)	117 ± 7	90.1 ± 3.0 #	111 ± 3	88.3 ± 3.6 #	*F*
Insulin (µg/l)	0.871 ± 0.087	0.321 ± 0.023 #	0.840 ± 0.101	0.396 ± 0.061 #	*F*
Leptin (µg/l)	4.05 ± 0.63	1.71 ± 0.23 #	4.27 ± 0.60	1.72 ± 0.26 #	*F*
Triglycerides (g/l)	1.83 ± 0.24	1.85 ± 0.05	1.91 ± 0.22	1.67 ± 0.32	
NEFA (mmol/l)	0.694 ± 0.089	0.888 ± 0.114	0.751 ± 0.097	0.957 ± 0.049	*F*

Circulating glucose, insulin, leptin, triglycerides and non-esterified fatty acid (NEFA) in male and female offspring of controls and caloric restricted dams during gestation (CR) at the age of 6 months, under *ad libitum* feeding conditions and after 12 h fasting conditions. Data are mean±S.E.M. (n=6-8). Statistics: R, effect of caloric restriction; F effect of fasting (two-way ANOVA). *, different from their respective control group (Student's t test); #, different from fed conditions (Student's t test).

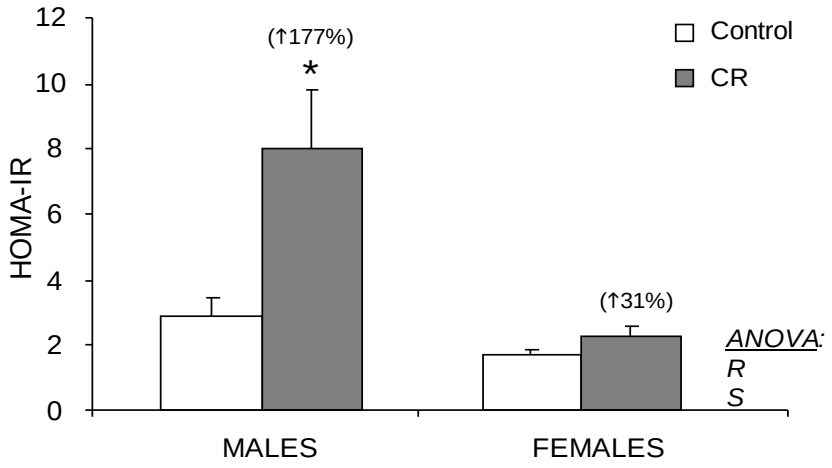

Figure 4. HOMA-IR index at the age of 6 months of male and female offspring of controls and caloric restricted dams during gestation (CR). Results are expressed as the mean ± SEM of 6-8 animals per group. The percentage increase in HOMA-IR index of CR compared with controls is indicated in brackets. Statistics: R, effect of caloric restriction, and S, effect of sex, ($p<0.05$, two-way ANOVA). *, different from their respective control group ($p<0.05$; Student's t test).

Gene expression levels in hypothalamus, retroperitoneal WAT and liver under fed and fasting conditions

Hypothalamic mRNA levels of selected genes involved in energy balance in control and CR male and female rats under fed and fasting conditions is shown in Figure 5. In male animals (Figure 5A), no significant differences were found concerning mRNA expression levels of Insr, Obrb, Npy, or Pomc as an effect of caloric restriction or fasting conditions (two-way ANOVA). However, it should be highlighted that the resulting Npy/Pomc mRNA ratio increased in control animals after fasting conditions (Student's t test), but was unchanged in CR male animals. With regard to females (Figure 5B), CR animals showed altered gene expression of hypothalamic key genes; in concrete, these animals showed lower Obrb and Pomc mRNA levels than their controls, with no changes in Insr and Npy (two-way ANOVA). In addition, CR females showed increased Pomc mRNA levels under fasting conditions (Student's t test), while no significant changes were found in control animals. Moreover, as occurring in males, the resulting Npy/Pomc mRNA ratio increased in control animals after fasting conditions (Student's t test), but was unchanged in CR female animals.

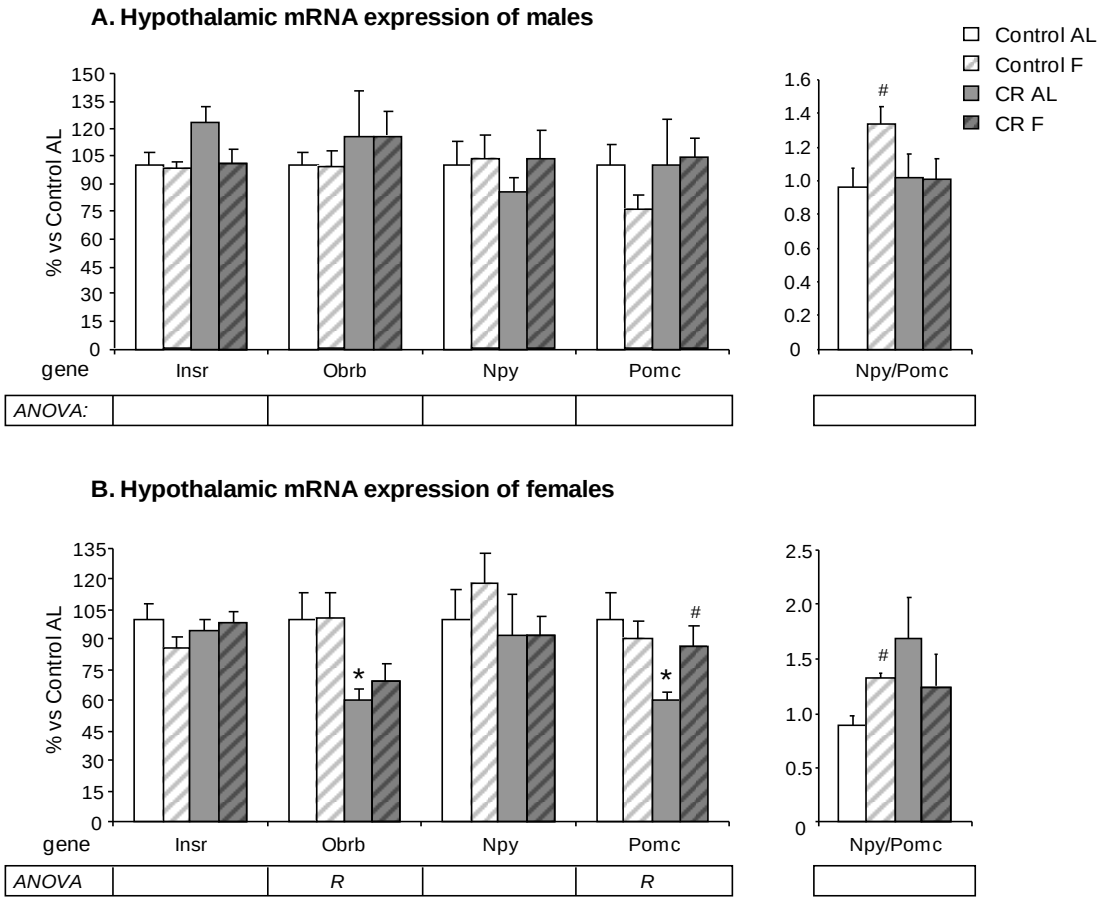

Figure 5. mRNA expression levels of insulin receptor (Insr), long form leptin receptor (Obrb), neuropeptide Y (Npy) and proopiomelanocortin (Pomc), and the Npy/Pomc ratio in the hypothalamus of male (A) and female (B) offspring of controls and caloric restricted dams during gestation (CR), at the age of 6 months, under ad libitum feeding (AL) and fasting (F) conditions. mRNA levels were measured by Real-time PCR and expressed as a percentage of the mean value of the control group under ad libitum feeding conditions. Data are mean SEM (n = 6-8). Statistics: R, effect of caloric restriction ($p<0.05$, two-way ANOVA). *, Control vs. CR; and #, Ad libitum vs. Fasting ($p<0.05$, Student's t test).

Figure 6 shows mRNA expression levels of selected genes related with energy balance in rWAT of control and CR male and female rats. Although different adipose tissue depots were harvested, gene expression analyses were performed in the retroperitoneal depot, based on the literature showing that this depot seems to be more sensitive to nutritional status, compared with other depots (Palou et al. 2010c). CR male animals showed lower Insr and Obrb mRNA levels than their controls (two-way ANOVA) (Figure 6A). In addition, concerning Insr, their mRNA levels increased in control animals under fasting conditions, but did not change in CR animals as an effect of food deprivation. CR animals also showed higher Tnfα mRNA expression levels under fasting conditions in comparison to fasted control rats (Student's t test). Moreover CR male animals showed a different response to fasting conditions concerning Cpt1 expression levels: they increased in control animals under fasting conditions, but not in

CR animals. In addition, CR male animals showed lower Cpt1 mRNA levels under fasting conditions than their controls (Student's t test). Figure 6B shows gene expression in rWAT of female animals. Interestingly, CR female animals, under fed conditions, showed lower Obrb mRNA levels than their controls. CR animals also showed higher Tnfα expression levels compared to controls (two-way ANOVA). In addition there was a different response to fasting conditions between control and CR animals concerning Atgl mRNA expression (interaction between caloric restriction and fasting, two-way ANOVA), since mRNA levels increased in control animals under fasting conditions, but levels did not change in CR animals. In fact, CR female animals under fasting conditions showed lower Atgl mRNA levels than their controls (Student's t test). No significant differences were found between control and CR female animals concerning Insr mRNA levels.

Liver mRNA levels of selected genes related with energy balance in control and CR male and female rats are shown in Figure 7. Interestingly, CR male animals showed lower Obrb mRNA expression levels under *ad libitum* fed conditions than their controls (Figure 7A). Fasting induced an increase in Obrb mRNA levels in both groups, although this was more pronounced and only significant by Student t test in CR rats (interactive effect between caloric restriction during pregnancy and food deprivation, two-way ANOVA). Both Srebp1c and Acc1 mRNA expression levels decreased under fasting conditions in control and CR male animals, but in both cases the response to starvation was of a greater magnitude in control animals. Concerning Gpat, a significant decrease was found in control animals as an effect of fasting, but no changes were found in CR animals.

The results of hepatic gene expression in females are shown in Figure 7B. Both control and CR females displayed higher Insr mRNA levels after fasting conditions (two-way ANOVA), but the increase was higher and significant (by Student's t test) in controls. Obrb mRNA expression levels also increased after fasting conditions in both groups (two-way ANOVA), but the response was higher in CR animals; threfore female CR rats showed higher Obrb mRNA levels under fasting conditions compared to controls (two-way ANOVA). Srebp1c, Acc1 and Gpat mRNA levels decreased in both control and CR female animals as an effect of fasting, while no significant differences were found between control and CR female animals concerning the expression of these genes.

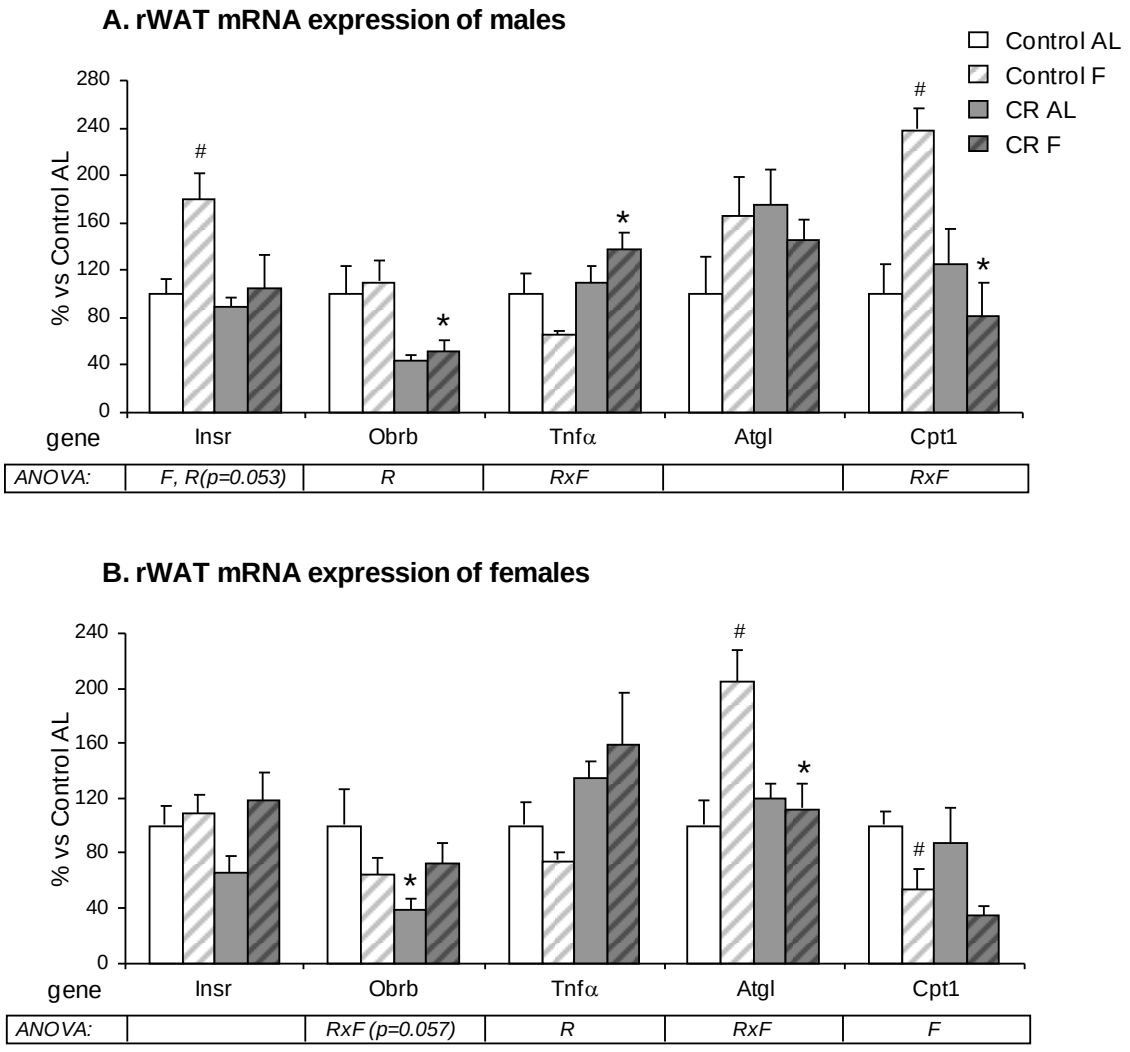

Figure 6. mRNA expression levels of insulin receptor (Insr), long form leptin receptor (Obrb), tumor necrosis factor alpha (Tnfα), adipose triglyceride lipase (Atgl) and carnitine palmitoyltransferase 1 (Cpt1) in rWAT of male (A) and female (B) offspring of controls and caloric restricted dams during gestation (CR), at the age of 6 months, under *ad libitum* feeding (AL) and 12-h fasting (F) conditions. mRNA levels were measured by Real-time PCR and expressed as a percentage of the mean value of the control group under *ad libitum* feeding conditions. Data are mean ± SEM (n = 6-8). Statistics: *R*, effect of caloric restriction; *F*, effect of fasting; and *RxF*, interaction between caloric restriction and feeding conditions ($p<0.05$, two-way ANOVA). *, Control *vs.* CR; and $^{\#}$, *Ad libitum vs.* Fasting ($p<0.05$, Student's t test).

Protein levels of insulin and leptin signaling molecules in rWAT and liver of control and CR male and female rats

Table 5 shows protein levels of IRS1, pIRS1, PKCζ, STAT3 and pSTAT3 in rWAT and liver of control and CR animals, under fed and fasting conditions. Compared to controls, CR male animals showed higher levels of total STAT3 in rWAT and lower PKCζ in liver. CR female animals showed lower levels of PKCζ in rWAT, and lower STAT3 (only under fed conditions) and pSTAT3 in liver. No changes were found concerning IRS1 and pIRS1 between controls and CR animals either in rWAT or liver.

Fasting reduced PKCζ and total STAT3 protein levels in rWAT of both controls and CR male rats (two-way ANOVA), while no changes were found in female animals as an effect of fasting, with the exception of a decrease in PKCζ levels in CR rats (Student's t test). In liver, fasting conditions resulted in lower pIRS1 (which was higher and significant by Student's t test in controls), PKCζ, STAT3 and pSTAT3 in males but, notably, it resulted in increased levels of IRS1, pIRS1 and of STAT3 in females (two-way ANOVA).

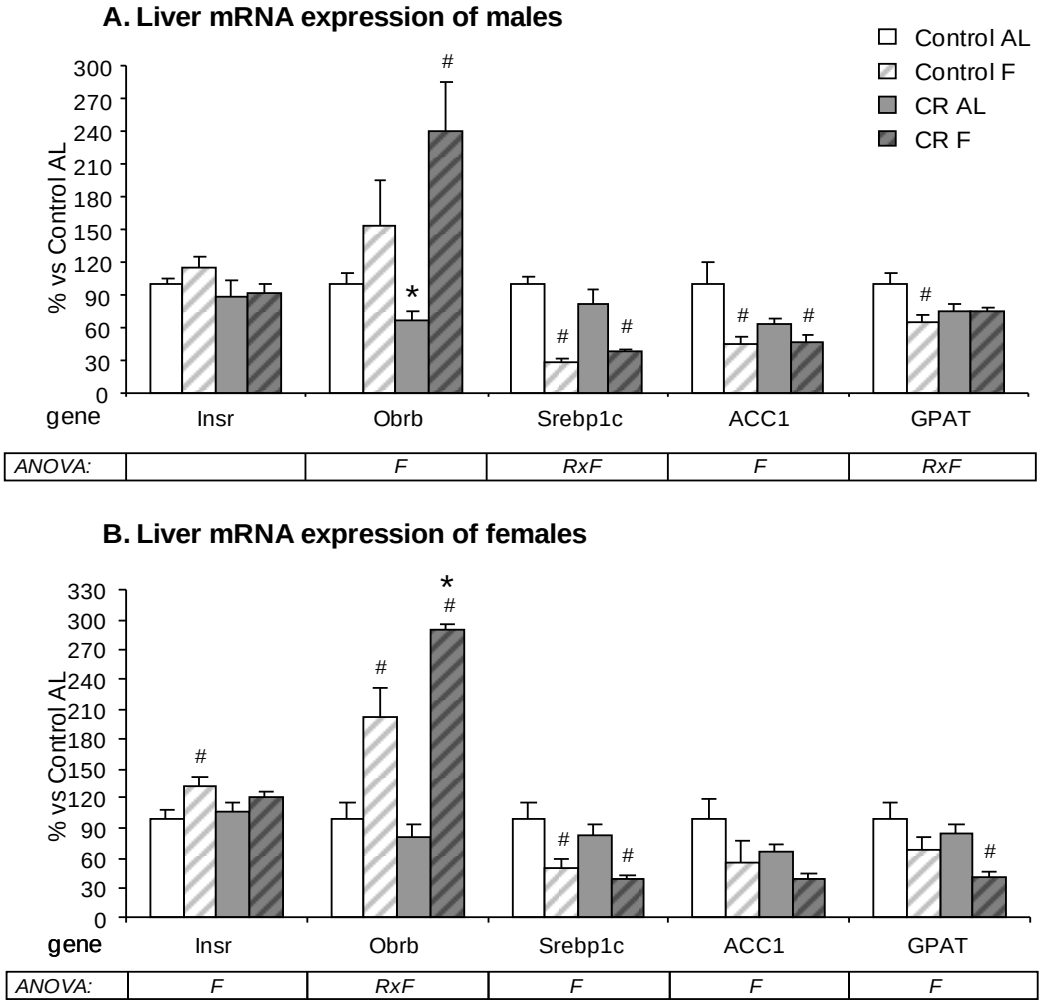

Figure 7. mRNA expression levels of insulin receptor (Insr), long form leptin receptor (Obrb), sterol response element binding protein 1c (Srebp1c), acetyl-coenzyme A carboxylase alpha (Acc1) and glycerol-3-phosphate acyltransferase (Gpat) in liver of male (A) and female (B) offspring of controls and caloric restricted dams during gestation (CR), at the age of 6 months, under *ad libitum* feeding (AL) and 12-h fasting (F) conditions. mRNA levels were measured by Real-time PCR and expressed as a percentage of the mean value of the control group under *ad libitum* feeding conditions. Data are mean ± SEM (n = 6-8). Statistics: *F*, effect of fasting; and *RxF*, interaction between caloric restriction and feeding conditions ($p<0.05$, two-way ANOVA). *, Control *vs.* CR; and $^{\#}$, *Ad libitum vs.* Fasting ($p<0.05$, Student's t test).

Table 5. Protein levels of insulin and leptin signalling molecules in the retroperitoneal WAT and liver.

	rWAT					Liver				
	Control		CR			Control		CR		
	Ad Libitum	12-h fasting	*Ad Libitum*	12-h fasting	*ANOVA*	*Ad Libitum*	12-h fasting	*Ad Libitum*	12-h fasting	*ANOVA*
Males										
IRS1	100 ± 18	112 ± 22	154 ± 33	133 ± 32		100 ± 8	94.7 ± 6.1	89.1 ± 4.9	110 ± 11	
pIRS1 (Tyr632)	100 ± 18	116 ± 4	246 ± 95	217 ± 76		100 ± 10	56.1 ± 6.4 #	98.4 ± 11.1	70.6 ± 6.3	*F*
PKCζ	100 ± 7	87.1 ± 14.7	96.2 ± 4.0	67.9 ± 11.7	*F*	100 ± 6	82.4 ± 6.1	84.6 ± 4.7	72.0 ± 1.8 #	*R,F*
STAT3	100 ± 20	83.9 ± 11.1	141± 12	110 ± 17	*R,F*	100 ± 13	57.2 ± 14.3	102 ± 26	56.4 ± 13.2	*F*
pSTAT3 (Tyr705)	100 ± 29	88.5 ± 10.2	161± 43	145 ± 42		100 ± 14	58.1 ± 11.5 #	75.2 ± 11.8	56.1 ± 9.7	*F*
Females										
IRS1	100 ± 22	108 ± 16	81.3 ± 22,5	94.6 ± 19.5		100 ± 5	119 ± 15	113 ± 9	151 ± 13 #	*F*
pIRS1 (Tyr632)	100 ± 24	101 ± 17	106 ± 11	93.0 ± 14.1		100 ± 5	157 ± 13 #	106 ± 17	157 ± 7 #	*F*
PKCζ	100 ± 9	123 ± 16	93.0 ± 10.7	58.4 ± 10.0 *#	*R*	100 ± 10	90.3 ± 10.4	90.9 ± 8.5	89.1 ± 7.3	
STAT3	100 ± 13	87.5 ± 25.8	163± 33	128 ± 28		100 ± 11	144 ± 14 #	61.1 ± 10.6 *	215 ± 61	*F*
pSTAT3 (Tyr705)	100 ± 18	98.1 ± 35.3	71.6 ± 8.9	111 ± 23		100 ± 25	118 ± 9	59.4 ± 16.2	76.2 ± 12.1 *	*R*

Protein levels in the retroperitoneal WAT (rWAT) and the liver of male and female offspring of controls and caloric restricted dams during gestation (CR) at the age of 6 months, under *ad libitum* feeding conditions and after 12 h fasting conditions. Data are mean±S.E.M. (n=4-6). Statistics: R, effect of caloric restriction; F effect of fasting (two-way ANOVA). *, different from their respective control group (Student's t test); #, different from fed conditions (Student's t test).

Discussion

In agreement with our previous results in the same cohort of animals when younger (Palou et al. 2010a), we show here that moderate maternal caloric restriction of 20% during the first half of pregnancy programs the offspring for higher food intake, which results in higher body weight and higher body fat content in males but not in females. We further show here that the hyperphagia displayed by these animals may be related with early programming of central and peripheral insulin resistance, and of central leptin resistance, and it is associated with gender-dependent changes in the expression profile of key genes involved in the control of energy homeostasis in adult rats. In addition, it is suggested that the lack of a circulating leptin surge during the suckling period in the offspring of caloric restricted animals during gestation may be one of the mechanisms that contribute to the metabolic malprogramming effects on target organs.

Fetal programming of insulin and leptin resistance by nutritional conditions has been proposed as a major mechanism responsible for later energy homeostasis dysregulation (Lustig et al. 2004; Esteghamati et al. 2009). In agreement with this, the results obtained here in adult rats exposed to HF diet concerning plasma leptin levels in males, and the HOMA-IR index in both males and females, but particularly in males, also suggest an impairment of insulin and leptin sensitivity in CR rats, which may explain their hyperphagia. It should be mentioned that although changes in leptin levels are found here in male animals under the stressor of HF diet, an increase in HOMA-IR in both males and females was already found in the same cohort of animals when younger and exposed to a NF diet (Palou et al. 2010a).

The effects of caloric restriction during gestation in male animals resulted in higher body weight and adiposity in adulthood compared to their controls. In addition, CR male adult animals also displayed an altered circulating TG response to feeding conditions, since these animals did not exhibit a decrease in their circulating TG levels after 12-h fasting, while it occurred in control animals. Repeated exposure of the liver to elevated insulin levels has been described to induce hepatic TG production (Zammit 2002). In contrast, a better blood TG profile has been related with an improvement of insulin and leptin sensitivity (Palou et al. 2011b). Hence, the dysregulation of circulating metabolic parameters in CR male rats is in agreement with the development of insulin and leptin resistance.

Unlike males, adult CR females did not display significant changes in circulating insulin and leptin levels compared to their controls. However, these animals presented other alterations related with insulin and leptin signaling at the central and peripheral level (see below), but they seem to be partially protected against the excess of fat accumulation associated with HF-diet feeding. CR female animals only showed greater size of the gonadal fat pad weight compared with their controls, but no significant changes were found in the other fat depots studied. In addition, they seem to be able to maintain normal levels of circulating parameters, such as TG levels. These results suggest that female animals are moderately protected against the detrimental effects of maternal caloric restriction during gestation. Jones and Friedman (Jones and Friedman 1982) also observed sex-differences in the offspring of 50% caloric-restricted dams during the first 2 weeks of pregnancy, where male rats gained more weight after 5 weeks of age and became obese, but female offspring did not develop obesity. The reasons for the sex-dependent different outcomes in adult body weight and adiposity of fetal undernutrition are not clearly elucidated yet. However the decrease in adipose tissue sympathetic innervation described in male offspring of caloric restricted dams during gestation, but not in females, could account for the different outcomes on later adiposity (Garcia et al. 2011).

To ascertain whether the manifestation of insulin and leptin resistance seen in the adult offspring of caloric restricted animals during gestation was secondary to age and/or the obesogenic diet challenge or was the result of metabolic programmed effects, the expression of insulin and leptin receptors in selected tissues and circulating hormone levels were also studied in 25-day old animals. At this age, these animals did not display significant changes in body weight, and no changes were found concerning insulin levels between control and CR animals either. However circulating leptin levels were lower in CR animals compared with controls, and these changes do not seem to be attributed to lower body weight or adiposity. However, interestingly, at the age of 25 days, CR animals showed altered expression levels of insulin and leptin receptors in key target organs. Specifically, CR animals displayed lower mRNA expression levels of insulin receptor in the three tissues studied, the hypothalamus, rWAT and the liver, and lower mRNA levels of leptin receptor in the hypothalamus, with no significant changes in rWAT or liver.

The hormones insulin and leptin are able to exert their function at the central level,

directly to the hypothalamus, through the regulation of the expression of different neuropeptides and key factors involved in energy homeostasis maintenance (Schwartz et al. 2000). Hypothalamic alterations affecting the expression levels of insulin and leptin receptors already seen in CR animals at the age of 25 days suggest a malprogramming of the central control of appetite behavior. In agreement with these findings, we have previously described that both male and female offspring of caloric restricted rats during gestation had, at the early age of 25 days of life, lower NPY and αMSH-producing neurons and lower total cells in the arcuate nucleus, which were accompanied by altered mRNA expression levels of these neuropeptides, as well as increased expression levels of Socs-3, which could be indicative of lower central sensitivity to leptin and insulin (Garcia et al. 2010).

We show here that adult CR animals exposed to HF diet for a 2 month period did not exhibit apparent differences in Insr mRNA levels in the hypothalamus compared with their controls. The lack of significant differences in Insr mRNA levels between control and CR animals when adult, while seen at early stages of life, could be tentatively attributed to the detrimental effects of HF feeding that may mask the effects of the early exposure to caloric restriction. In fact, HF diet has been described to contribute to central insulin resistance (De Souza et al. 2005). Concerning Obrb mRNA levels, the early differences between control and CR animals observed at the age of 25 days were maintained at the age of 6 months but only in females. Again, the lack of differences in male animals could be attributed to the overlapping effects of HF diet in these animals, whereas females could be more protected against the detrimental effects of this dietary challenge, according to the literature (Priego et al. 2008; Priego et al. 2009).

In addition, CR adult animals showed an altered response to fed/fasting conditions, concerning mRNA expression levels of Npy and Pomc. The Npy/Pomc ratio rose in both male and female control animals under fasting conditions, but such changes were not observed in CR rats, suggesting a decreased capacity of the CNS to sense and respond to changes in nutrient availability. This may contribute to explain the higher food intake of CR rats. Previous studies have also shown that obesity is characterized by an impaired response to feeding conditions, which has been observed both when studying the mRNA expression and protein levels of a number of genes involved in energy metabolism (Caimari et al. 2007; Pico et al. 2002) as well as for hundreds of genes measured by transcriptomics in peripheral tissues (Caimari et al. 2010a; Caimari

et al. 2010b). Moreover, it is interesting to highlight that CR females, which also exhibited lower Obrb mRNA expression, also showed lower Pomc mRNA levels than their controls, which would be closely involved in their dysregulated food intake. A decrease in the mRNA expression levels of Obrb has been described to be involved in the development of leptin resistance (Baskin et al. 1998). Therefore, central insulin and leptin resistance appear to be early malprogramming effects of moderate caloric restriction during gestation, which may be responsible for impaired food intake control and hence hyperphagia in both male and female CR animals.

Although the hypothalamus is the central controller of appetite behavior and energy balance, the peripheral response to both circulating insulin and leptin hormones may also determine the susceptibility to develop obesity and other related metabolic alterations. In this sense, WAT is a key tissue involved in both fat accumulation and mobilization, and these processes are regulated, among others hormones, by insulin and leptin; alterations in the action of these hormones due to malprogramming effects on early life (Ikenasio-Thorpe et al. 2007; Vickers et al. 2000) or in their adaptability under obesogenic environments such as HF diet feeding may affect the propensity to develop overweight (Priego et al. 2008). At the age of 25 days of life, CR rats already presented lower WAT Insr mRNA levels, with no changes in Obrb expression levels, but a tendency to lower levels. When adult and under HF diet, CR males, but not females, also displayed lower Insr mRNA levels and both male and female CR animals presented lower Obrb mRNA levels than their controls.

Levels of proteins involved in insulin and leptin signaling in adult animals have been measured as they may provide evidence of the presumed altered sensitivity to these hormones. Results obtained for PKC are supportive as CR female animals showed lower levels of PKCζ compared with their controls. This decrease was more marked under fasting conditions. PKCζ is a downstream effector in the insulin signaling pathway and plays an important role in activating the glucose transport response; in fact, overexpression of PKCζ or constitutively active PKCζ has an insulin-like effect on glucose transport during *in vitro* incubation of different kinds of cell lines [rev. (Liu et al. 2006)]. Thus, the decrease in PKCζ protein levels in rWAT of CR females suggests a decrease in insulin signaling and glucose uptake by the adipose tissue. Decreased PKCζ levels in insulin target organs, such as muscle, have also been found in different experimental models of undernutrition during gestation and related with major

propensity to insulin resistance development in adulthood (Chen et al. 2009).

However, despite changes in PKCζ in female CR animals, the protein levels of total IRS1 or in pIRS1 were not significantly different between control and CR animals. In the same way, STAT3 and pSTAT3 levels did not decrease in CR animals either; conversely, CR male animals showed higher protein concentration of total STAT3 in this tissue, but no differences in the pSTAT3/STAT3 ratio. The higher protein levels of STAT3 in CR males could be the result of their hyperleptinemia, which allows certain leptin signaling in these animals. IRS1 plays a critical role in insulin signaling being the initial step, while STAT3 mediates leptin signaling (Morris and Rui 2009). Leptin is also able to induce an insulin-like signaling pathway involving IRS/PI3K and making this a relevant point of cross-talk between the insulin and leptin signaling pathways [rev. (Fruhbeck 2006)]. Protein levels of pIRS1 have been found altered in other models of maternal malnutrition during gestation. In particular, the offspring of 60% protein restricted mice during gestation showed, at the age of 21 days, lower levels of pIRS1 and of other insulin signaling proteins in muscle, suggesting a predisposition of these animals to insulin resistance (Chen et al. 2009). Here, although no effect of maternal caloric restriction during gestation was observed in IRS1 protein levels in adult animals, we cannot rule out that other key molecules of the insulin signaling cascade could be affected. In addition, as previously discussed, we cannot rule out either that the negative effects of HF diet on insulin resistance could be masking the effect of these maternal conditions during gestation.

Even though changes in proteins involved in insulin and leptin signaling in adult CR animals under HF diet did not prove a decreased adipose tissue sensitivity to these hormones, compared with controls, it is interesting to highlight that both male and female CR rats showed higher expression levels of the proinflammatory cytokine TNFα. TNFα is elevated in adipose tissue in many rodent models of obesity and affects insulin sensitivity (Hotamisligil et al. 1993). In fact a link between obesity, insulin resistance and inflammation has been proposed (Gual et al. 2005). TNFα has been involved in the JNK mediated serine phosphorilation of IRS1, which inhibits the normal tyrosine phosphorilation of IRS1 in response to insulin (Gual et al. 2005). In adult CR males, obesity was clearly manifested, but CR females did not present overweight at that moment of life, but these results, in accordance with other alterations in the expression of other key genes in WAT, such as the Obrb gene, suggest a dysregulation

of energy homeostasis in these animals. Since females have been described to be more protected against the detrimental effects associated to obesity compared to males (Priego et al. 2008) and CR females presented severe alterations in other factors involved in the control of energy balance and appetite behavior at the central and peripheral level, it could be thought that CR females might be in a previous stage of the disease and could develop excessive fat accumulation, together with other endocrine alterations, later on in life. In favor of the detrimental programming effects of maternal caloric restriction during gestation in the female offspring is the lack of increase in Atgl mRNA expression levels in rWAT under fasting conditions, which occurs in control animals, in agreement with the literature (Palou et al. 2008); this could be indicative of an impaired capacity to mobilize TG from this tissue under a negative energy balance situation. On the other hand, and in accordance with their higher fat accumulation in the adipose tissue, CR male animals did not exhibit an increase in Cpt1 mRNA levels under fasting conditions, while this increase was found in control animals.

Regarding the liver, at the age of 25 days, CR rats presented lower Insr mRNA levels and no significant changes in Obrb mRNA levels compared to controls. Interestingly, when adult, and under HF diet, CR males showed lower Obrb mRNA levels than controls, but only under fed conditions, with no changes in Insr mRNA levels. Nevertheless, CR males also showed lower PKCζ protein levels than their controls. In addition, although hepatic levels of IRS1 and STAT3 did not change between control and CR animals, it must be noted that their phosphorilated levels (Tyr 632 and Tyr 705, respectively) showed a diminished response to fasting conditions compared with their controls. All in all, these results agree with impaired leptin and insulin signaling in liver, which could be associated with the impaired response to changes in fed/fasting conditions concerning lipogenesis. In fact, CR males displayed a lower or even a lack of response to fasting conditions concerning the expression levels of Srebp1c and Gpat, respectively. Unlike males, no changes were found in females between control and CR animals concerning the expression of insulin and leptin receptors, or of other genes related with lipid metabolism. CR females even showed higher Obrb mRNA levels than their controls under fasting conditions. This does not seem to be indicative of higher leptin signaling in these animals, since they displayed lower protein levels of total STAT3 (only under fed conditions) and of their phosphorylated (Tyr705) form, which suggests lower leptin signaling. The function of leptin in liver is not clearly established,

but it has been shown to have antisteatotic effects by lowering the mRNA expression of Srebp1. In fact, mice with ablated hepatic leptin signaling have increased lipid accumulation in the liver (Kakuma et al. 2000). Thus, leptin appears to act as a negative regulator of insulin action in liver (Huynh et al. 2010), and therefore impaired leptin action in liver may affect whole energy homeostasis.

Thus, these results concerning the expression levels of insulin and leptin receptors in peripheral tissues such as WAT and liver at early stages of life, together with the apparent impaired action of these hormones found in adult life, suggests that early programming of peripheral insulin resistance may be a direct consequence of fetal caloric restriction, while peripheral leptin resistance, which appears in adulthood and under HF diet, might be secondary to insulin resistance or to central leptin resistance. In fact it is recognized that elevated insulin levels promote both insulin resistance and increased leptin biosynthesis and secretion from adipose tissue, which may further desensitize leptin signaling and increase leptin resistance (Seufert 2004). However, although the leptin-obesity-insulin resistance link is established, which alterations are causes or consequences in this particular situation and why the phenotypic outcomes are different between males and females needs further clarification.

All in all, we show here that maternal caloric restriction during gestation results in early effects on the expression of leptin and insulin receptors in key tissues involved in energy homeostasis, particularly hypothalamus, which may compromise the proper functioning of the leptin and insulin systems. Moderate caloric restriction has also been associated with malprogramming of central hypothalamic structures involved in energy balance (Garcia et al. 2010) and with a reduction in adipose tissue symphatetic innervations (Garcia et al. 2011). However, the mechanisms or factors responsible for the detrimental effects of these perinatal conditions are not clearly established. Leptin, which is naturally present in maternal milk, is known to play a key role during the suckling period (Palou and Pico 2009). Leptin supplementation (physiological doses) in rats during the suckling period has later effects in the offspring preventing overweight in adulthood (Pico et al. 2007) as well as other alterations related with the metabolic syndrome (Priego et al. 2010), and also improves later insulin and leptin sensitivity (Sanchez et al. 2008). These beneficial effects of leptin during lactation seem to be related, at least in part, with a better control of food intake, associated with epigenetic changes in the promoter methylation of Pomc (Palou et al. 2011a). Moreover, in

rodents, it is known that plasma leptin levels rise transiently during neonatal period, peaking around day 10 of lactation, a process that has been termed as "neonatal leptin surge" (Ahima et al. 1998; Rayner et al. 1997; Pico et al. 2011). This surge in leptin levels seems to be important for programming the structural and functional development of hypothalamic orexigenic and anorexigenic centers (Grove and Smith 2003) and its potential alteration by maternal caloric restriction has been checked here. In control animals we observed the expected peak in circulating leptin levels at this period (9 days of life), but it is suggestive that this was absent in CR animals. A premature or delay in leptin peak, as occurring in mice with severe fetal undernutrition (Yura et al. 2005) or in protein restricted rats during gestation and during both gestation and lactation (Zambrano et al. 2006), has also been associated with obesity in adulthood. Nevertheless, whether the malprogramming effects observed by caloric restriction could be simply attributed to the lack of the leptin surge needs to be specifically addressed. In any case, these results underscore the importance of leptin during lactation and the critical consequences that leptin deficiency may have during a critical period in postnatal life, being responsible for the common, detrimental outcomes of different adverse perinatal conditions.

In conclusion, results show that 20% maternal caloric restriction during the first 12 days of gestation programs the offspring for a lower capacity to respond to insulin and to central leptin action, which is already present at early ages, and this leads to hyperphagia in both genders and higher body weight in males but not in females. Males show higher and earlier harmful effects by caloric restriction during fetal life than females, while females appear more resistant to the detrimental effects of gestational caloric restriction, in terms of maintenance of body weight, in spite of the altered profile of gene expression in key tissues involved in energy homeostasis. The lack of leptin surge during a critical window of developmental plasticity, such as the suckling period, appears closely associated with the adverse health effects observed in the offspring of caloric restricted dams.

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MANUSCRIPT 2

Moderate calorie restriction during gestation programs offspring for lower BAT thermogenic capacity driven by thyroid and sympathetic signaling

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Title page

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Abstract

Maternal calorie restriction during pregnancy programs offspring for later overweight and metabolic disturbances. Brown adipose tissue (BAT) is responsible for nonshivering thermogenesis and has recently emerged as a very likely target for human obesity therapy. Here we aimed to assess whether the detrimental effects of undernutrition during gestation could be related to impaired thermogenic capacity in BAT and to investigate the potential mechanisms involved. Offspring of control and 20% calorie-restricted rats (days 1-12 of pregnancy) (CR) were studied at the age of 25-days. Protein levels of uncoupling protein 1 (UCP1) and tyrosine hydroxylase (TyrOH); mRNA levels of lipoprotein lipase (Lpl), carnitine palmitoyltransferase 1 (Cpt1) and deiodinase iodothyronine type II (Dio2) in BAT; and blood parameters including thyroid hormones, were determined. The response to 24-h cold-exposure was also studied by measuring body temperature changes over time, and final BAT UCP1 levels. Compared to controls, CR animals displayed in BAT lower UCP1 and TyrOH protein levels and lower Lpl and Cpt1 mRNA levels; they also showed lower triiodothyronine (T3) plasma levels. CR males, but not females, revealed lower Dio2 mRNA levels than controls. When exposed to cold, CR rats experienced a transient decline in body temperature, but the values were reestablished after 24-h, despite having lower UCP1 levels than controls. These results suggest that BAT thermogenic capacity is diminished in CR animals, involving impaired BAT sympathetic innervation and thyroid hormone signaling. These alterations make animals more sensitive to cold and may contribute to long-term outcomes of gestational calorie restriction in promoting obesity and related metabolic alterations.

Keywords: calorie restriction, pregnancy, thermogenesis, brown adipose tissue, sympathetic innervation, thyroid hormones, cold response

Introduction

Nutritional environment during sensitive periods of early development is thought to program later body weight. The hypothesis of fetal origins of adult diseases, proposed by Barker and collaborators (Godfrey and Barker 2000), suggests that poor fetal nutrition leads to metabolic adaptations programming subjects to be more sensitive to obesity development under a positive nutritional environment. The Dutch Famine epidemiological study showed that the incidence of obesity was higher in men whose mothers underwent malnutrition during the first 6 months of pregnancy due to the acute famine suffered in western Holland during World War II (Ravelli et al. 1976). In animal models, severe (30 to 50%) maternal calorie restriction during gestation has been associated to obesity predisposition and other related alterations, such as insulin resistance (Anguita et al. 1993; Jones and Friedman 1982; Jones et al. 1984; Vickers et al. 2000). More recently, less severe maternal calorie restriction during gestation (20%) has also been described to program lasting detrimental effects in offspring, but in a sex dependent manner: effects on body weight maintenance were particularly found in males, although both genders displayed lower insulin and leptin sensitivity accompanied by hyperphagia (Palou et al. 2010). We have described some mechanisms that may account for the effects on food intake (Garcia et al. 2010) and white adipose tissue (WAT) (Garcia et al. 2011), but the potential involvement of brown adipose tissue (BAT) has not been explored.

Early programming of adipose depots has been described to be determinant for the later functionality of the tissue (Priego et al. 2013; Symonds et al. 2011). In mammals, besides WAT, there are small amounts of BAT, which is the main site of adaptive thermogenesis, providing extra heat in hibernating animals, newborns and cold-exposed mammals (Tam et al. 2012). Brown adipocytes, unlike the white ones, are characterized by multilocular lipid droplets and a greater amount of mitochondria that express uncoupling protein 1 (UCP1) (Sell et al. 2004; Cinti 2005). UCP1 uncouples the rates of substrate oxidation and ATP production by favoring a loss of protons and energy release in the form of heat (Palou et al. 1998). In addition, BAT is also important in the control of energy balance since it is activated in response to diet (Cannon and Nedergaard 2004). Therefore, activation of BAT thermogenesis represents a mechanism that enables dissipating, in a regulated manner, part of the energy from food as heat instead of accumulating it as fat. The recent recognition of BAT presence and function in adult

humans has promoted a renewed interest in BAT thermogenesis as a possible target for stimulation of energy expenditure to help mitigate increased body fat storage (Nedergaard et al. 2007).

The sympathetic innervation of BAT is the most significant physiological effector of the thermogenic process. This is triggered by the release of norepinephrine (NE) that turn on a cascade of intracellular events ending in activation of UCP1, together with other processes, including triglyceride (TG) mobilization, thus providing the fatty acids necessary for UCP1 activation (Palou et al. 1998; Cannon and Nedergaard 2004). Thyroid hormones also play a key role in the activation of adaptive thermogenesis. In concrete, triiodothyronine (T3) is necessary for the full expression of UCP1 and amplifies the adrenergic stimulation of Ucp1 mRNA expression in cold-exposed rats (Silva 2006). T3 also increases the adrenergic stimulation of BAT deiodinase, iodothyronine, type II (DIO2) (Martinez de Mena et al. 2010) a selenoenzyme that locally produces T3, via 5' deiodination of thyroxine (T4).

Given the contribution of BAT to energy homeostasis and its potential interest as a target for obesity therapy, here we aimed to assess whether impaired BAT thermogenesis capacity could also contribute to adverse outcomes of maternal calorie restriction during gestation in relation to a higher propensity to develop overweight and related metabolic alterations, and, if so, to ascertain which mechanisms could be responsible for this alteration.

Methods

The animal protocol followed in this study was reviewed and approved by the Bioethical Committee of our University and guidelines for the use and care of laboratory animals of the University were followed.

Study 1

Animals and experimental design

The study was performed in 25 day-old male and female rats from 29 different litters, including the offspring of control and 20% calorie-restricted rats during the first part of gestation, as previously described (Palou et al. 2010). All rats were housed under controlled temperature (22 ºC) and a 12 h light–dark cycle (light on from 0800 to 2000),

and had unlimited access to tap water and standard chow diet (3 kcal/g, with 8% calories from fat; Panlab, Barcelona, Spain) unless mentioned otherwise. Briefly, virgin female Wistar rats weighing between 200 g and 250 g were mated with male rats (Charles River Laboratories, Barcelona, Spain). Day of conception (day 0 of pregnancy) was determined by examination of vaginal smears for the presence of sperm, and then female rats were single caged. Pregnant rats were divided into two groups: one with free access to standard chow diet (n=13), and the other one underwent 20% restriction of calorie intake from day 1 to day 12 of pregnancy (n=16). Calorie restriction was performed by offering each dam, at the beginning of the light phase, a daily amount of food corresponding to 80% of the calories that should be eaten according to body weight. This amount was calculated considering the calories consumed daily by their control animals under *ad libitum* feeding conditions. After the calorie restriction period, rats were allowed to eat *ad libitum*. On day 1 after delivery, excess pups in each litter were removed to keep 10 pups per dam (five males and five females, when possible). Weaning was conducted on postnatal day 21.

At the age of 24 days body weight of pups was recorded. On day 25 of life and during the first 2 h of the beginning of the light cycle, one group of animals from control dams and from calorie-restricted dams (CR) was killed by decapitation after 12-h fasting (n= 6-8 animals/group) and a second group of rats was killed under ad libitum feeding conditions (n= 5-8 animals/group). Blood samples were collected from the neck in heparinized containers. Plasma was obtained by centrifugation of blood at 1000 g for 10 min and stored at -20ºC until analysis. Interscapular BAT was rapidly removed, weighed and frozen in liquid nitrogen and stored at -80ºC until subsequent studies. Tissue analyses were conducted in BAT of animals under basal (12-h fasting) conditions.

Measurement of circulating parameters

Blood glucose concentration was measured by Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Insulin concentration was determined using ultrasensitive rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia AB, Uppsala, Sweden), and leptin concentration was measured using ELISA kit QuantikineTM Mouse Leptin Inmunoassay (R&D Systems, Minneapolis, MN, USA). Thyroid-stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4)

circulating levels were analyzed by using the commecial Elisa kits EIA-1780 (T3), EIA-1781 (T4) and EIA-5296 (TSH) (DRG, Marburg, Germany).

Western blot analysis

The amount of UCP1 and tyrosine hydroxylase (TyrOH) in BAT of control and CR rats was determined by western blot. Tissue was homogenized at 4 ºC in 1:10 (w:v) of Phosphate buffer saline (PBS). The homogenate was centrifuged at 500 g for 10 min at 4 ºC and the supernatant was used for protein analysis. Total protein content was measured by the method of Bradford (Bradford 1976). For analysis, 40 μg of total protein was solubilized and boiled for 3 min in Laemmli sample buffer containing 5% 2-beta-mercaptoethanol. Then, total protein was fractionated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE; 10 % polyacrylamide), and electrotransferred onto nitrocellulose membrane (Bio-Rad, Madrid, Spain). Black amide B10 staining provided visual evidence for correct loading and blotting of proteins. After blocking, the membrane was incubated with the primary rabbit polyclonal anti-UCP1 (GeneTex, Inc., CA, USA) or anti-TyrOH (Santa Cruz Biotechnology, Inc., CA, USA), and then with the infrared (IR)-dyed secondary anti-IgG antibody (LI-COR Biociences, Nebraska, USA) diluted 1:25000. For IR detection, membranes were scanned in Odyssey Infrared Imaging System (LI-COR Biociences, Nebraska, USA), and the bands were quantified using the analysis software provided.

RNA extraction

Total RNA was extracted from BAT by EZNA® TOTAL RNA kit I (Omega Bio-Tek Inc., Norcross, GA, USA) according to the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies Inc., Wilmington, Delaware, USA) and its integrity confirmed using 1% agarose gel electrophoresis.

Real-time quantitative PCR (RT-qPCR) analysis

Real-time polymerase chain reaction (PCR) was used to measure mRNA expression levels of deiodinase, iodothyronine, type II (Dio2), lipoprotein lipase (Lpl) and carnitine palmitoyltransferase 1 beta (Cpt1) in BAT. 0.25 μg of total RNA (in a final volume of 5 μl) was denatured at 65 ºC for 10 min and then reverse transcribed to cDNA using

MuLV reverse transcriptase (Applied Biosystems, Madrid, Spain) at 20ºC for 15 min, 42ºC for 30 min, with a final step of 5 min at 95ºC in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Madrid, Spain). Each PCR was performed from diluted cDNA template, forward and reverse primers (1 μM each), and Power SYBER Green PCR Master Mix (Applied Biosystems, Madrid, Spain). Real time PCR was performed using the Applied Biosystems StepOnePlusTM Real-Time PCR Systems (Applied Biosystems, Madrid, Spain) with the following profile: 10 min at 95ºC, followed by a total of 40 two-temperature cycles (15 s at 95ºC and 1 min at 60ºC). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.1) and the relative expression of each mRNA was calculated as a percentage of male control rats, using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001); beta-actin and Gdi1 were used as reference genes. Sequences of primers (obtained from Sigma, Madrid, Spain) were: β-actin, forward: 5'-TACAGCTTCACCACCACAGC-3', reverse: 5'-TCTCCAGGGAGGAAGAGGAT-3'; Gdi1, forward: 5'-CCGCACAAGGCAAATACATC-3', reverse: 5'-GACTCTCTGAACCGTCATCAA-3'; Dio2, forward: 5'-TTCTCCAACTGCCTCTTCCT-3', reverse: 5'-CAGGTCGCTGAACCAAAGT-3'; Lpl, forward: 5'-TATGGCACAGTGGCTGAAAG-3', reverse: 5'-CTGACCAGCGGAAGTAGGAG-3'; Cpt1, forward: 5'-GCAAACTGGACCGAGAAGAG-3', reverse: 5'-CCTTGAAGAAGCGACCTTTG-3. Stable expression of reference genes was checked. The maximum difference between Ct averages of the different groups was 0.400 (for beta-actin) and 0.266 (for GDI1).

Study 2

In view of the results obtained in the first study, in a second step we analyzed the response to cold exposure of controls and CR rats. For this, a new cohort of controls and CR rats from 8 different litters (4 controls and 4 calorie-restricted dams) was obtained as described above. At the age of 24 days, controls and CR rats were exposed to cold (6ºC) for 24 hours and rectal temperatures were recorded at specific time points: 0, 1-h, 2-h, 4-h and 24-h. Temperatures were recorded from 15-18 rats per group. On day 25, after the 24-h cold exposure period, a set of animals from each group (control and CR) was killed (n=8-10 rats/group) and interscapular BAT was rapidly collected for western

blot analyses of UCP1. Western blot was performed as described for study 1, but using 10 μg of protein.

Statistical analysis

Data are expressed as means ± S.E.M. Two-way, three-way or repeated measures analysis of variance (ANOVA) with the factors of calorie restriction (R), sex (S), fasting (F) or cold exposure (C) was performed to assess statistical significances. Individual means were compared with a Student's t test or Paired t test. The analyses were performed with SPSS for Windows (SPSS, Chicago, IL). Threshold of significance was defined at $p<0.05$.

Results

Study 1

As previously described in another group of animals (Garcia et al. 2010), control and CR animals exhibited no significant differences in their body weight at the age of 24 days, or in the weight of BAT at sacrifice (data not shown).

Blood parameters are shown in Table 1. 12 hours of fasting caused a significant decreased in glucose, insulin and leptin in all groups of animals ($p<0.05$, two-way ANOVA). No significant differences were found in glucose, insulin, leptin, TSH or T4 circulating levels due to maternal calorie restriction during gestation. However, CR rats showed lower plasma levels of the T3 hormone ($p<0.05$, two-way ANOVA), with the differences being more marked and significant by Student's t test ($p<0.05$) only in males.

Figure 1A shows specific protein levels of UCP1 in BAT of male and female control and CR rats. CR animals revealed lower UCP1 levels than controls ($p<0.05$, two-way ANOVA).

Specific protein levels of TyrOH in BAT are shown in Figure 1B. CR animals also showed lower TyrOH protein levels than their controls ($p<0.05$, two-way ANOVA), although the decrease was more pronounced and only significant by Student's t test ($p<0.05$) in CR males.

Gene expression levels of Dio2 are shown in Figure 1C. Notably, a sex-dependent effect of maternal calorie restriction during gestation was found (interactive effect between

calorie restriction and sex, $p<0.05$, two-way ANOVA); while CR males showed lower Dio2 mRNA levels compared to controls ($p<0.05$, Student's t test), no significant differences were found among females.

Figure 2 shows mRNA levels of Lpl and Cpt1 in control and CR rats. Expression levels of both genes were lower in CR animals compared to controls ($p<0.05$, two-way ANOVA). The decrease in Lpl mRNA expression levels was more marked and only significant by Student's t test ($p<0.05$) in CR males.

Table 1. Study 1: Circulating parameters

		Males		Females		
		Control	CR	Control	CR	ANOVA
Glucose (mg/dl)	Fed	148 ± 4 (8)	145 ± 2 (7)	149 ± 4 (7)	148 ± 5 (7)	F
	Fasting	76 ± 4 (8)	78 ± 5 (6)	64 ± 3 (6)	59 ± 5 (6)	
Insulin (ng/l)	Fed	170 ± 24 (8)	204 ± 33 (7)	248 ± 41 (7)	240 ± 49 (7)	F
	Fasting	40± 6 (6)	48 ± 8 (6)	52 ± 5 (6)	47 ± 9 (6)	
Leptin (ng/l)	Fed	2206 ± 170 (8)	1798 ± 121 (7)	1689 ± 241 (7)	1461 ± 145 (7)	F
	Fasting	40 ± 24 (6)	17 ± 8 (6)	27 ± 13 (6)	78 ± 51 (6)	
TSH (ng/ml)	Fed	1.18 ± 0.07 (8)	1.14 ± 0.08 (7)	1.47 ± 0.23 (7)	1.23 ± 0.12 (5)	
T3 (ng/ml)	Fed	5.64 ± 0.37 (8)	4.13 ± 0.45 * (7)	5.63 ± 0.58 (7)	4.54 ± 0.35 (5)	R
T4 (ng/ml)	Fed	45.4 ± 2.3 (8)	49.4 ± 1.6 (7)	45.9 ± 0.6 (7)	46.3 ± 2.4 (5)	

Blood levels of glucose, insulin and leptin under fed and fasting conditions and of thyroid-stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4) under fed conditions of male and female offspring from controls and calorie-restricted dams during gestation (CR) at the age of 25 days. Data are mean±S.E.M. The number of animals per group is indicated in brackets. Statistics: F, effect of fasting ($p<0.05$, three-way ANOVA); R, effect of calorie restriction ($p<0.05$, two-way ANOVA); *, CR *vs* Control ($p<0.05$; Student's t test).

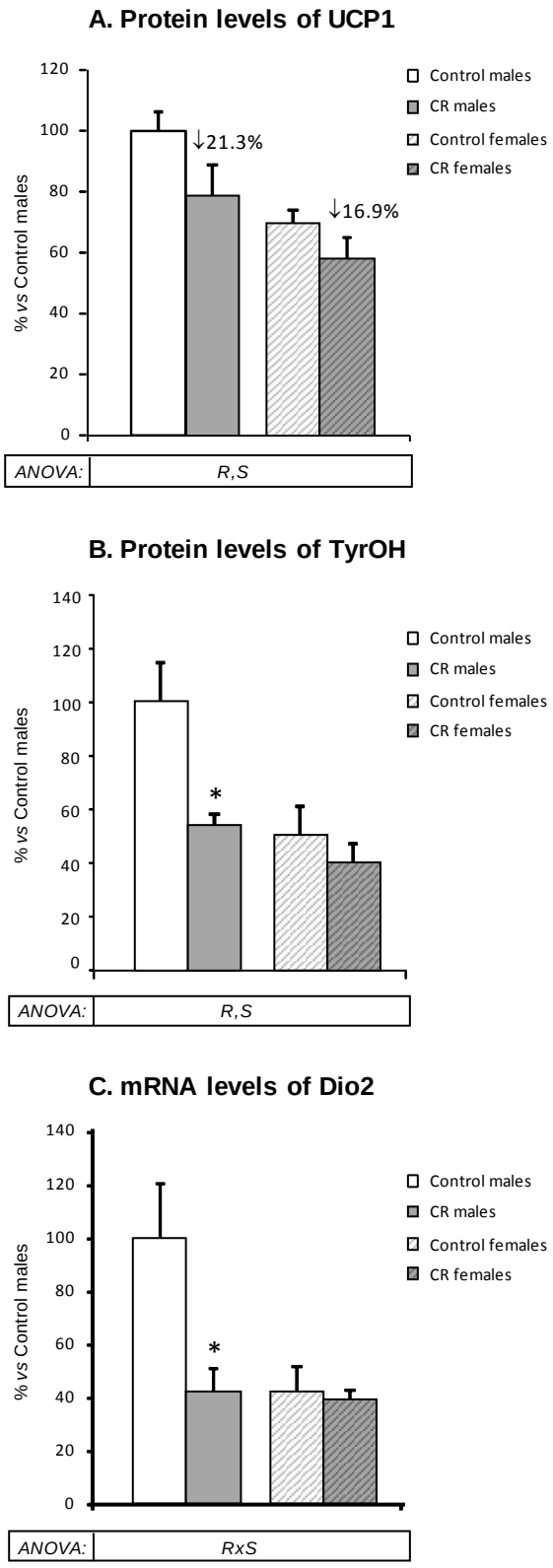

Figure 1. Specific abundance of uncoupling protein 1 (UCP1) (A) and of tyrosine hydroxylase protein (TyrOH) (B), and mRNA levels of deiodinase, iodothyronine, type II (Dio2) (C), in brown adipose tissue of 25-day-old male and female offspring from control and calorie-restricted dams during gestation (CR). Data are expressed as a percentage of the mean value of control male rats. Data are mean ± S.E.M. (n=6-8). Percentages of decrease of UCP1 levels in CR rats with respect to controls are indicated. Statistics: R, effect of calorie restriction; S, effect of sex; and RxS, interactive effect between calorie restriction and sex ($p<0.05$, two-way ANOVA). *, CR *vs* Control ($p<0.05$; Student's t test).

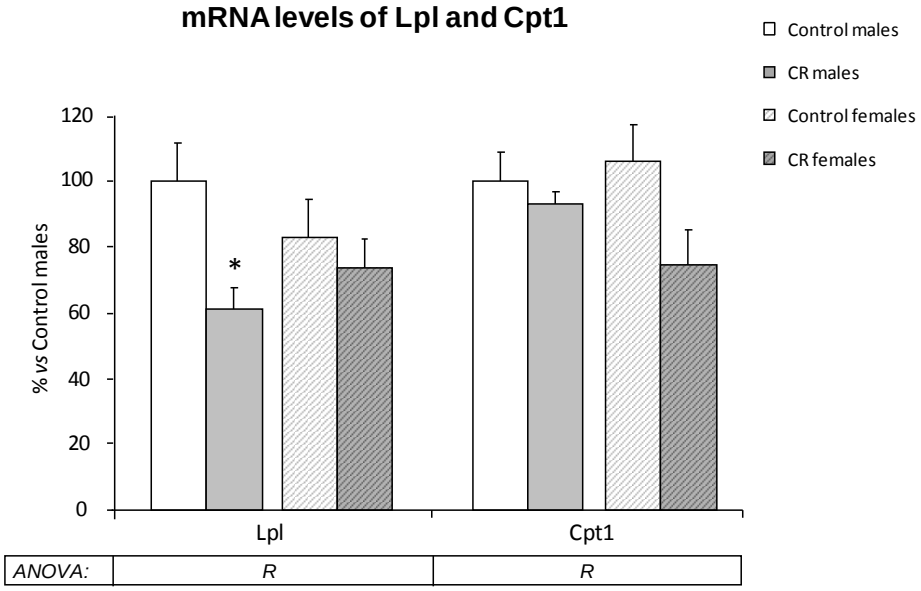

Figure 2. mRNA levels of lipoprotein lipase (Lpl) and carnitine palmitoyltransferase 1 b (Cpt1) in brown adipose tissue of 25-day-old male and female offspring from control and calorie-restricted dams during gestation (CR). Data are expressed as a percentage of the mean value of control male rats. Data are mean ± S.E.M. (n=6-8). Statistics: R, effect of calorie restriction ($p<0.05$, two-way ANOVA). *, CR *vs* Control ($p<0.05$; Student's t test).

Study 2

Table 2 shows the weight-related parameters of controls and CR animals belonging to the second study. In this study, both male and female CR animals already showed higher body weight than their controls at the age of 24 days ($p<0.05$, two-way ANOVA). The reason for the difference from Study 1 is not known; this could be potentially attributed to particular characteristics of the different cohorts of animals, which could have caused a more prompt effect on body weight gain of CR animals than in Study 1. Of note, no significant differences were found at birth between controls and CR rats in either study (data not shown). In this study, it was shown that both male and female control animals increased their body weight over the 24-hours-cold exposure period, while body weight of CR animals remained practically unchanged ($p<0.05$, two-way ANOVA). No significant differences were found in the weight of BAT between control and CR animals at sacrifice.

Table 2. Study 2: Weight-related parameters.

	Males		Females		
	Control (8)	CR (10)	Control (8)	CR (10)	ANOVA
Body weight (g)	58.7 ± 2.0	65.5 ± 1.1 *	58.1 ± 1.7	61.9 ± 1.2	R
Body weight gain (g)	1.66 ± 0.29	0.367 ± 0.349 *	1.41 ± 0.64	-0.446 ± 0.613	R
Body weight gain (%)	2.72 ± 0.38	0.579 ± 0.532 *	2.53 ± 1.16	-0.636 ± 0.954 *	R
BAT weight (mg)	159 ± 6	175 ± 10	155± 6	165 ± 7	

Body weight (at the age of 24 days, before cold exposure), body weight gain during the 24-h period of cold exposure and weight of brown adipose tissue (BAT) at sacrifice (day 25) of male and female offspring from control and calorie-restricted dams during gestation (CR). Data are mean±S.E.M. The number of animals per group is indicated in brackets. Statistics: R, effect of calorie restriction ($p<0.05$, two-way ANOVA). *, CR vs Control ($p<0.05$; Student's t test).

Figure 3 shows body temperature during cold exposure in control and CR rats. In males (Figure 3A), cold exposure caused a significant reduction in body temperature in the CR group after one and two hours, but they partially recovered their normal temperature after 4 hours and totally after 24 hours ($p<0.05$, Paired t test); in contrast, control rats maintained their temperature throughout the whole period studied. After 2 hours of cold exposure CR male animals showed a trend to lower temperature compared to controls ($p= 0.061$, Student's t test), and an interactive effect between maternal calorie restriction and body temperature over time during cold exposure ($p<0.05$, ANOVA repeated measures) was found. A different response to cold was also found between control and CR female animals (interactive effect between maternal calorie restriction and body temperature over time, $p<0.05$, ANOVA repeated measures) (Figure 3B). CR female rats showed a decrease in their body temperature during the first 4 hours of cold exposure ($p<0.05$, Paired t test), although they managed to recover it at 24 hours. On the other hand, control females maintained their temperature during the first 2 hours, but displayed a significant reduction in their temperature at 4 hours ($p<0.05$, Paired t test), which was reestablished after 24 hours.

Specific UCP1 protein levels in BAT of control and CR animals after 24-h cold exposure are shown in Figure 4. Under these conditions, both male and female CR rats also revealed lower UCP1 levels than controls ($p<0.05$, two-way ANOVA), with the differences being more marked than those observed at 22ºC.

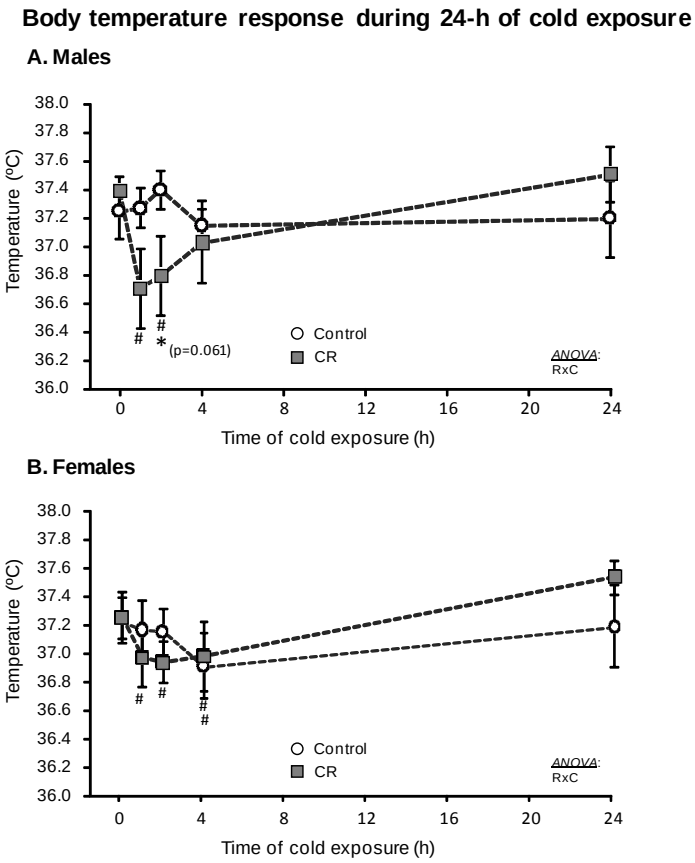

Figure 3. Body temperature of male and female offspring from control and calorie-restricted dams during gestation (CR) over 24 hours cold exposure (from day 24 to day 25 of life), measured at different time-points: before cold exposure (T0) and after 1- (T1), 2- (T2), 4- (T4) and 24-h (T24). Data are mean ± S.E.M. (n=11-18). Statistics: RxC, interactive effect between calorie restriction and the time-response to cold ($p<0.05$, ANOVA repeated measures). $^{\#}$, T1 or T2 or T4 or T24 *vs* T0 ($p<0.05$, Paired t test); and *, CR *vs* Control ($p=0.061$, Student's t test).

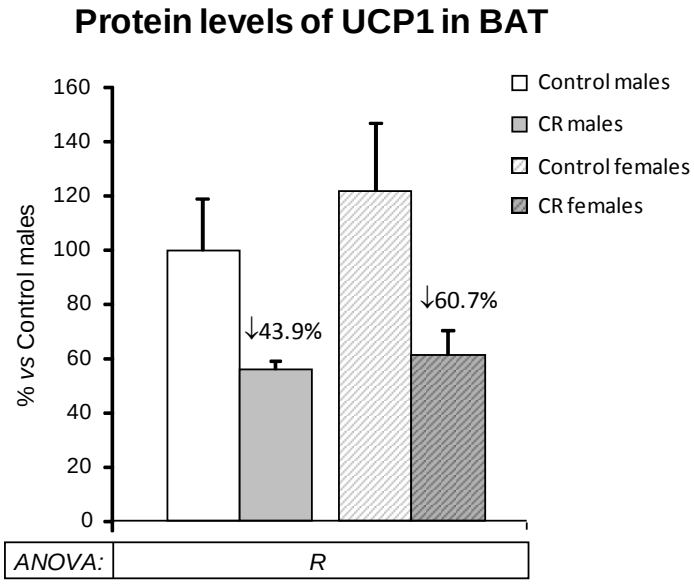

Figure 4. Specific abundance of uncoupling protein 1 (UCP1) in brown adipose tissue of 25-day-old male and female offspring from control and calorie-restricted dams during gestation (CR) after 24 hours cold exposure. Data are expressed as a percentage of the mean value of control rats. Data are mean ± S.E.M. (n=6). Percentages of decrease of UCP1 levels in CR rats with respect to controls are indicated. Statistics: R, effect of calorie restriction ($p<0.05$, two-way ANOVA).

Discussion

The presence and activity of BAT in adult humans has reopened the interest for this tissue as a target for obesity therapy and other metabolic-related pathologies, such as insulin resistance and type 2 Diabetes (Tam et al. 2012; Nedergaard and Cannon 2010). Research aimed at ascertaining which factors and conditions during early stages of life may program BAT function and activity, and hence contribute to later effects on the capacity to maintain body weight, is becoming of great interest.

We previously showed that moderate (20%) maternal calorie restriction during gestation programs lasting detrimental effects in offspring, particularly obesity, insulin resistance and alterations in other risk factors of cardiovascular disease and diabetes (Palou et al. 2010). Potential mechanisms involved in the programming effects of this nutritional condition during pregnancy still remain to be fully elucidated. Here we show for the first time that 20% maternal calorie restriction during the first part of gestation results in the offspring in a significant reduction of specific UCP1 protein levels in BAT: 21.3% and 16.9% in males and females, respectively, compared to their controls. Although we have not directly measured energy expenditure or BAT functioning, the reduction of UCP1 levels might be interpreted as a decreased thermogenic capacity, although this is an indirect measure. The lower burning capacity of BAT could contribute, at least in part, to the greater predisposition to fat accumulation and other metabolic alterations that CR animals suffer, particularly males, in adulthood (Palou et al. 2010). The acute activity of BAT, such as heat production, and the recruitment process in the tissue are under the control of NE released from sympathetic nerves (Palou et al. 1998; Nedergaard et al. 1995). To ascertain whether the reduced thermogenic capacity of CR rats could be due to an impairment in sympathetic innervation, the sympathetic drive was estimated by measuring TyrOH protein levels, the rate-limiting enzyme in catecholamine synthesis (Giordano et al. 1996). Protein content measurement of this enzyme has been described to be a good estimation of sympathetic innervations (Giordano et al. 2005). Interestingly, we found that CR animals exhibited lower TyrOH levels in BAT; the decrease occurred in both males and females, although it was only significant by Student's t test in males (45.9% decrease compared to their respective controls). Notably, a significant positive correlation between UCP1 and TyrOH protein levels in BAT was also found ($p=0.040$). This is in agreement with the relationship between sympathetic innervation and UCP1 production in BAT, therefore suggesting

that the reduced thermogenic capacity could be due, at least in part, to impaired sympathetic signaling. The alterations in sympathetic innervation occurring in CR animals do not seem to be exclusive of BAT. We previously described, in a different group of animals, that male offspring of moderate calorie-restricted rats during gestation, but not females, showed lower iWAT sympathetic innervations (Garcia et al. 2011). Hence, it could be speculated that an impairment in WAT and BAT sympathetic innervation may contribute to the detrimental effects of maternal calorie restriction during gestation in offspring; sex-dependent differences in the occurrence and magnitude of the alteration may in turn account for the previously described sex-dependent outcomes in adult body weight and adiposity (Palou et al. 2012).

The lower burning capacity of BAT occurring in CR animals was found to be associated with lower mRNA expression levels of genes related to fuel supply and fatty acid oxidation in this tissue. CR animals displayed lower Lpl and Cpt1 mRNA levels, compared to controls. LPL activity in BAT increases the supply of fatty acids from circulating lipoproteins for metabolic needs and maintenance of thermogenesis. When TG stored in BAT are not enough to maintain heat production, extra lipids have to be imported into the tissue; LPL is the enzyme responsible for the delivery of fatty acids from circulating lipoproteins (Nedergaard et al. 1995). Unlike what occurs in WAT, LPL activity and expression in BAT is under the control of NE (Mitchell et al. 1992). Thus, the decrease in mRNA levels of Lpl found in CR animals, particularly in males, may be reflecting the alterations in sympathetic innervation occurring in these animals, which may in turn lead to a lower fuel supply for thermogenic activity. Although LPL present in BAT has generally received less attention compared to that present in other peripheral tissues, such as WAT and skeletal muscle, changes in the amount or activity of this enzyme in BAT may also contribute to plasma clearance of TG (Bartelt et al. 2011). In this regard, we previously described that both male and female young adult CR animals displayed higher circulating TG levels than their controls (Palou et al. 2010). Meanwhile, CPT1 catalyzes the limiting rate for mitochondrial β-oxidation, constituting the major pathway whereby fatty acids are oxidized to generate energy (Kerner and Hoppel 2000). The observed reduced Cpt1 mRNA expression levels in BAT of CR rats amount to a likely metabolic profile in BAT where a lower capacity for fatty acid uptake accompanies a limited fatty acid oxidation capacity.

Thyroid hormones also play a fundamental role in BAT activity (Lowell and Spiegelman 2000). The control of thermogenesis by NE is under the action of the thyroid hormones T3 and thyroxine (T4). T4 is converted to the active form T3 by the deiodinase iodothyronine enzymes (Williams and Bassett 2011). Thyroid hormones interact with the SNS, being critical for the full activation of NE signaling cascade and full thermogenesis activation in BAT (Silva 2006). In fact, the disability of hypothyroid rodents to activate BAT thermogenesis by NE can be solved by the administration of T3 or T4 (Ribeiro et al. 2000; Ribeiro et al. 2001). DIO2 is also an essential component in the thyroid-sympathetic synergism required for thermal homeostasis in small mammals (de Jesus et al. 2001). The main role of DIO2 is to regulate intracellular T3 concentration, its accessibility to the nucleus, and the saturation of the nuclear T3 receptor in target tissues (Williams and Bassett 2011). Transgenic mice with a disruption of Dio2 gene exhibit dramatically impaired thermogenesis in BAT and a greater predisposition to diet-induced obesity, in spite of having normal plasma T3 concentration (de Jesus et al. 2001; Hall et al. 2010).

Therefore, the SNS is essential for the activation of BAT thermogenesis, although its responsiveness is dependent on the concentration of T3 in the tissue, which in turn depends on the DIO2 activity that is stimulated by NE (Silva 2006). In the present study, we found that CR animals, and particularly males, presented lower plasma T3 levels and lower BAT Dio2 expression levels. The reduced concentration of the active form of thyroid hormones is not only in agreement with the greater propensity to develop overweight of these animals, since hypothyroidism has been related to higher fat accumulation (Longhi and Radetti 2013), but also with reduced UCP1 production. It could be propounded that the decreased T3 plasma levels occurring in CR animals, together with the lower expression levels of Dio2 in BAT, may impair NE signaling in this tissue, which is impaired *per se* due to maternal conditions during gestation, and altogether contribute to the reduced burning capacity of CR animals. It is noteworthy that the reduction in Dio2 mRNA levels was only found in CR males, and they also displayed a more dramatic decrease in sympathetic drive to BAT in comparison to females. This sex-dependent response is in agreement with the major reduction in T3 plasma levels occurring in males and their proneness to fat accumulation later in life, as previously described (Palou et al. 2010).

All in all, the results obtained so far suggest that CR animals have an imprinted impaired BAT thermogenic capacity compared to controls. To ascertain whether this situation occurring in CR animals could affect their thermoregulation capacity, we evaluated body temperature during a 24h period of cold exposure (Study 2). Interestingly, CR animals, both males and females, showed a deficient capacity to maintain body temperature during the first hours of cold exposure, but they managed to restore their normal temperature after 4h (at least partially, in the case of males) or 24h in the case of females. Control rats, particularly males, presented a better capacity to maintain body temperature during the period studied. These results suggest that thermoregulatory capacity is diminished in CR rats, although they can manage to maintain body temperature after an acclimatizing period, at least during short periods of cold exposure as studied here.

Increased UCP1 production does not seem to be responsible for the recovery of normal body temperature after 24h of cold exposure. CR animals showed lower UCP1 levels in BAT than controls. The difference between control and CR animals was more exacerbated (decreases of 43.9% and 60.7% in male and female animals, respectively) compared to what found at 22ºC in Study 1. This suggests that cold-induced BAT thermogenesis is also impaired in CR rats. Maintenance of body temperature in CR animals found at 24h of cold exposure may be tentatively attributed, at least in part, to a greater activation of shivering thermogenesis. It is known that when unacclimatized animals are placed in a cold environment, they acutely defend their body temperature by activation of both BAT thermogenesis (also referred as non-shivering thermogenesis) and shivering thermogenesis in skeletal muscle. It has been estimated that the latter may provide around one-third of the total heat production during cold exposure, being very important in the maintenance of homeothermy (Florez-Duquet and McDonald 1998). However, this form of thermogenesis consumes large amounts of energy and interferes with normal activity (Silva 2006). In agreement with this, body weight gain in CR animals during the 24 hours period of cold exposure was negligible or even tended to decrease, and was in any case significantly lower compared to that of controls. This suggests that CR animals exhibited greater activation of the shivering thermogenesis to counterbalance the deficient activation of BAT (non-shivering) thermogenesis. Alternative explanations could also be possible. Maintenance of body temperature in CR animals could be achieved by increased voluntary locomotor activity to combat cold

exposure, and/or by increased metabolic rate or futile cycling in other organs than BAT, which is supported by the absence of an increase in body weight during the 24h cold exposure. Hence, although the exact contributing mechanism is not known, results suggest that the difference between short-term (<4h) and long-term (24h) response to cold observed between control and CR animals might involve BAT and non-BAT effects. All in all, our results support the fact that CR animals have defective BAT thermogenesis, associated with a delayed thermal response to cold and to inefficient thermoregulation, and hence probably making these animals more sensitive to cold. Whether these results can be extrapolated to human is not known, but in view of the described presence of active BAT in humans and its relation with obesity (Nedergaard and Cannon 2010), present findings may give some cues for the early programming mechanisms of later diseases, particularly regarding energy dysbalance in adulthood, and hence merits to be investigated.

In summary, it can be outlined that moderate (20%) maternal calorie restriction during the first 12 days of pregnancy programs offspring for lower BAT thermogenic capacity at the juvenile age of 25 days, which may determine a greater sensitivity to cold and contribute to the higher propensity for fat accumulation and other metabolic alterations later in life. Both impaired sympathetic drive and altered thyroid hormone signaling in BAT, which occur particularly in males, are proposed as mechanisms contributing to the reduced BAT burning capacity. These results pinpoint the relevance of BAT activity programming in the control of later body weight and energy metabolism and also highlight the importance of adequate maternal nutrition during critical periods of development to establish later metabolic health in the offspring, since even moderate interventions may cause a myriad of malprogramming effects.

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MANUSCRIPT 3

Oral leptin treatment in suckling rats ameliorates detrimental effects in hypothalamic structure and function caused by maternal caloric restriction during gestation

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Title page

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Conflict of interest: A. Palou, C. Picó and J. Sánchez are authors of a patent held by the University of the Balearic Islands on the use of leptin as an essential nutrient during lactation. The patent referred in the manuscript is held by the University of the Balearic Islands. The name of the patent is "Use of leptin for the prevention of excess body weight and composition containing leptin" (WO 2006089987 A1) (priority data: 23 Feb 2005). The authors are: A. Palou, C. Picó, P. Oliver, J. Sánchez, and O. Miralles. So far, the patent has been accepted in Spain, Russia and Mexico.

Abstract

A poor prenatal environment brings about perturbations in leptin surge and hypothalamic circuitry that program impaired ability to regulate energy homeostasis in adulthood. Here, using a rat model of moderate maternal caloric restriction during gestation, we aimed to investigate whether leptin supplementation with physiological doses throughout lactation is able to ameliorate the adverse developmental malprogramming effects exerted in offspring hypothalamus structure and function. Three groups of male and female rats were studied: the offspring of *ad libitum* fed dams (controls), the offspring of 20% calorie restricted dams during the first part of pregnancy (CR), and CR rats supplemented with physiological doses of leptin throughout lactation (CR-Leptin). Animals were sacrificed on postnatal day 25. Morphometric and immunohistochemical studies on arcuate (ARC) and paraventicular (PVN) nucleus were performed and hypothalamic expression levels of selected genes were determined. In CR males, leptin treatment restored, at least in part, the number of immunoreactive neuropeptide Y (NPY^+) cells in ARC, the total number of cells in PVN, hypothalamic Npy, cocaine- and amphetamine-regulated transcript (Cart) and suppressor of cytokine signalling-3 (Socs-3) mRNA levels, and plasma leptin levels, which were decreased in CR animals. CR-Leptin males showed higher hypothalamic long-form leptin receptor (Obrb) mRNA levels, compared to control and CR animals. In CR females, leptin treatment reverted the increased number of cells in ARC and cell density in ARC and PVN, and reduced hypothalamic Socs-3 mRNA expression to levels similar to controls. Leptin treatment also reverted the increased relative area of NPY^+ fibers in the PVN occurring in CR animals. In conclusion, leptin supplementation throughout lactation is able to revert, at least partly, most of the developmental effects on hypothalamic structure and function caused by moderate maternal caloric restriction during gestation, and hence making this metabolic malprogramming reversible to some extent.

Keywords: Leptin; gestational caloric restriction; hypothalamus; neuropepdide Y; obesity; metabolic programming

Introduction

Obesity is multifactorial in origin, but is mostly attributed to the interaction between environmental conditions and genetic predisposition (Loos and Bouchard 2003). However, it is becoming increasingly apparent that nutritional factors during early stages of development can lead to permanent programming of central and peripheral systems that regulate energy balance (Waterland and Garza 1999; Cottrell and Ozanne 2008; Sullivan and Grove 2010; Pico et al. 2012; Palou et al. 2010a; Palou et al. 2010b). Obesity is associated with severe morbidity and is increasing in prevalence, thus determination of strategies enabling its prevention starting in the early stages of life, as well as the potentialities to reverse early programmed effects of obesity-related metabolic disorders, become of crucial relevance.

A considerable number of studies have addressed the lasting consequences of an adverse prenatal environment on health outcomes in the offspring. In this view, maternal prenatal malnutrition, as well as low birth weight, have been described to be associated with obesity and metabolic syndrome in adulthood (Ravelli et al. 1976; Grino 2005; Barker et al. 1993; Jones and Friedman 1982). The first, renowned evidence in scientific literature supporting this proposal comes from an epidemiological study on the consequences of the Dutch Hunger Winter famine, near the end of World War II. This study demonstrated that males born to mothers who underwent malnutrition during the first 2 trimesters of gestation were more likely to be obese in adult life (Ravelli et al. 1976). In current day, the presence of maternal undernutrition during gestation does not appear to account for the increasing prevalence of obesity in children and adults in developed societies, but this phenomenon could better explain the increasing prevalence of obesity among people in developing countries, which undergo the transition from chronic malnutrition to adequate or excessive nutrition (Hales and Barker 2001; Yang and Huffman 2013). Animal studies leading to a better understanding of this association have pointed out that maternal food restriction during pregnancy is a risk factor increasing vulnerability to later obesogenic environmental stimuli (Vickers et al. 2000; Palou et al. 2010a; Pico et al. 2012). In particular, moderate caloric restriction during the first half of gestation in rats has been described to program the offspring for greater food intake as well as for insulin and leptin resistance, which results in higher body weight and body fat content in males but not in females (Palou et al. 2010a; Palou et al. 2012). Despite these observations, elucidation of the mechanisms responsible for these ‘developmental origins of health and disease’ is a topic of great concern and still remains unclear.

There is emerging evidence showing that specific hypothalamic areas that regulate food intake and energy expenditure may be particularly susceptible to permanent programming by the early nutritional and hormonal environment, which could influence the capacity to regulate energy homeostasis in adulthood. In this sense, we and others have evidenced that nutritional manipulations during perinatal period, such as protein restriction (Plagemann et al. 2000; Minana-Solis and Escobar 2011) or caloric restriction (Delahaye et al. 2008; Ikenasio-Thorpe et al. 2007; Garcia et al. 2010), modify hypothalamic structure and function. In particular, perinatal 50% food restriction reduces nerve fibers immunoreactive to beta-endorphin (a product of POMC) projecting from the arcuate nucleus (ARC) to the paraventicular nucleus (PVN) in neonate rats (Delahaye et al. 2008). In turn, more moderate maternal caloric restriction (20%) during pregnancy also perturbed hypothalamic ARC structure in weaned rats, by decreasing the presence of total number of cells, and particularly NPY-neurons (Garcia et al. 2010).

Leptin, an adipocyte-derived hormone, plays a central role regulating energy balance. It acts particularly at the hypothalamic ARC to attenuate hunger, by inhibiting orexigenic neuropeptides (such as NPY and AgRP) and stimulating anorexigenic ones (such as POMC and CART), as well as increasing energy expenditure (Schwartz et al. 2000; Arora and Anubhuti 2006). In addition to its role in the regulation of energy homeostasis in adults, during early steps of development, leptin has been shown to play a crucial neurotrophic role in programming hypothalamic circuit formation, particularly nerves projections from ARC to the other hypothalamic areas (Bouret and Simerly 2006). Neurodevelopment action of leptin appears to be restricted to the second week of life, which is coincident with a rise in circulating leptin levels**,** the so-called leptin surge (Bouret et al. 2004; Bouret and Simerly 2006). Severe perinatal maternal food restriction (50%) (Delahaye et al. 2008), or even moderate maternal caloric restriction (20%) during the first 12 days of gestation, which has been shown to determine alterations in hypothalamic circuitry (Palou et al. 2012), has been found to be associated with a drastic reduction or even absence of postnatal leptin surge in the offspring. In this regard, studies in rats have evidenced that leptin supplied by milk or as a water solution during the suckling period can be absorbed by the immature stomach (Casabiell et al. 1997; Oliver et al. 2002; Sanchez et al. 2005) and be transferred to the bloodstream (Casabiell et al. 1997; Sanchez et al. 2005). Therefore, maternal milk may substantially contribute to circulating leptin in neonate rats, at a time when the adipose tissue is still immature (Palou and Pico 2009). Hence, considering the neurotrophic role of leptin, it

could be speculated that leptin supplementation during a critical window of developmental plasticity may have the ability to reverse some of the neuroanatomical defects and other features of the obese phenotype associated with absence or alterations in neonatal leptin surge (Bouret et al. 2004; Bouret and Simerly 2006). Interestingly, daily intraperitoneal injections of pharmacological doses of leptin (10 mg/kg, between postnatal day 4 and 12) in leptin deficient mice (ob/ob) has been shown to rescue the development of disrupted ARC projections (Bouret et al. 2004). Vickers *et al.* (Vickers et al. 2005) also evidenced that daily leptin subcutaneous injection (2.5 µg/g/d) from postnatal day 3-13 in female pups born from 30% calorie-restricted dams during gestation, normalized calorie intake, locomotor activity, body weight, fat mass, and fasting plasma glucose, insulin and leptin concentrations in adulthood. The same treatment with pharmacological doses of leptin in early undernourished male pups conferred protection against obesity development, but only when animals were under HF diet (Vickers et al. 2008). Notably, we have also described that neonate male rats born from adequately nourished dams, and orally treated with physiological amounts of leptin throughout the suckling period are more resistant to age-related increases in body weight and diet-induced weight gain (Pico et al. 2007), displaying improved insulin and leptin sensitivity (Sanchez et al. 2008; Priego et al. 2010), and showing lower preference for fat-rich food in adulthood (Sanchez et al. 2008). Overall, these results point out the essential role of leptin during lactation in imprinting healthier metabolic responses in later life (Palou and Pico 2009). However, no studies have been conducted so far considering the ability of oral leptin supplementation at physiological doses during lactation to reverse early malprogramming effects in hypothalamus associated to poor prenatal conditions.

In the present study, we used an experimental rat model of moderate (20%) maternal caloric restriction during pregnancy, which is known to be associated with alterations in hypothalamic circuitry that program a higher propensity to develop obesity in the offspring, particularly in males (Palou et al. 2010a), to investigate whether oral supplementation with physiological doses of leptin throughout lactation is able to ameliorate or normalize developmental malprogramming of hypothalamus, which may be responsible, at least in part, for the adverse health outcomes later in life.

Materials and methods

Animals and Experimental Design

The animal protocol followed in this study was reviewed and approved by the Bioethical Committee of the University of the Balearic Islands (Resolution Number 1798. February 18th, 2009) and guidelines for the use and care of laboratory animals of the University were followed.

The study was conducted on male and female Wistar rat pups from 17 different litters following the protocol during pregnancy and lactation as is described below. Animals were housed under standard conditions, that is, controlled temperature (22 C), the normal 12-h light and 12-h dark cycle, free access to tap water and a standard laboratory rodent chow diet (3.3 kcal/g, with 8% calories from fat; Panlab, Barcelona, Spain), unless specified. Virgin female Wistar rats (body weight 217 g - 244 g) were mated with male rats (Charles River Laboratories, Barcelona, Spain). Day of conception (day 0 of pregnancy) was determined by examination of vaginal smears for the presence of sperm. Pregnant rats were divided into two groups: control dams (n=7 animals) fed *ad libitum* with standard chow diet, and calorie restricted dams (CR-dams) (n=10 animals) fed with 20% caloric restriction from day 1 to day 12 of gestation, as previously described (Palou et al. 2010a). After the calorie restriction period, all dams were fed *ad libitum*, and food intake was measured.

On day 1 after delivery, excess pups in each litter were removed to keep 10 pups per dam (five males and five females, when possible). Pups of both sexes born from CR-dams were randomly assigned into two groups: CR and CR-Leptin. CR-Leptin animals were supplemented, each day throughout lactation with an oral solution of recombinant murine leptin (PeproTech, London, UK) dissolved in water by using a pipette. The amount of leptin given to animals was progressively increased from 1 ng of leptin on day 1, to 43.8 ng of leptin on day 20 of life, as previously described (Pico et al. 2007). CR pups and the offspring of control dams (controls) received the same volume of the vehicle (water).

Pups were weaned at 21 days of life, and 35 pups from control group (18 males and 17 females), 34 from CR group (17 males and 17 females), and 33 from CR-Leptin group (17 males and 16 females) were housed in groups of two animals, and fed on a standard chow diet. Body length (from the tip of the nose to the anus) and body fat content (by EchoMRI-700^{TM}, Echo Medical Systems, LLC., TX, USA) were measured in all the animals when

animals were 25 days old. Body weight and food intake were recorded from weaning until the age of 25 days.

On day 25, pups were sacrificed by decapitation, during the first 2 h of the beginning of the light cycle, under fed conditions. Some of these animals (n = 10–11, per group) were used for gene expression analysis and the others (n = 6-8, per group) to perform morphometric and immunohistochemical analysis. Animals used for the different analysis were from at least six different litters.

Blood samples were collected in heparinized containers, then centrifuged at 1000 x *g* for 10 min to obtain the plasma, and stored at −20 C until analysis. For gene expression studies, the hypothalamus was rapidly removed, immediately frozen in liquid nitrogen and stored at −80 C until RNA analysis. For morphometric analysis, brain samples were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4) at 4 C for 24 h, then washed and stored in 0.1 M phosphate buffer (pH = 7.4) until posterior analysis.

Quantification of glucose, insulin and leptin concentration

Blood glucose concentration was measured by Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Plasma insulin concentration was determined using ultrasensitive rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia AB, Uppsala, Sweden). Leptin concentration in plasma and in stomach homogenates was measured using ELISA kit Quantikine^{TM} Mouse Leptin Inmunoassay (R&D Systems, Minneapolis, MN, USA) as previously described (Sanchez et al. 2005).

RNA extraction

Total RNA was extracted from hypothalamus and stomach using TRIpure Reagent (Roche Diagnostic Gmbh, Mannheim, Germany), in accordance to the manufacturer´s instructions. RNA yield was quantified on the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies, Wilmington, DE, USA) and its integrity confirmed using 1% agarose gel electrophoresis.

Real-time quantitative PCR analysis

Real-time polymerase chain reaction was used to measure mRNA expression levels of Agouti-related peptide (Agrp), cocaine- and amphetamine-regulated transcript (Cart),

neuropeptide Y (Npy), long-form leptin receptor (Obrb), proopiomelanocortin (Pomc), and suppressor of cytokine signalling-3 (Socs-3) in hypothalamus and leptin mRNA levels in stomach. qRT-PCR analysis was performed as previously described (Palou et al. 2012). Primer sequences and products for the different genes are described in Table 1. All primers were purchased from Sigma Genosys (Sigma Aldrich Quımica SA, Madrid, Spain). In order to verify the purity of the products, a melting curve was produced after each run. The values for the threshold (Ct) were calculated by the instrument's software (StepOne Software v2.2.2), and the relative expression of each mRNA was calculated as a percentage of male control rats, using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001) with β-actin and rho gdp dissociation inhibitor alpha (GDI) (in hypothalamus) and 18S ribosomal (in stomach) as reference genes.

Table 1. Nucleotide sequences of primers and amplicon size used for qRT-PCR.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon (bp)
18S	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC	219
β-actin	TACAGCTTCACCACCACAGC	TCTCCAGGGAGGAAGAGGAT	120
Agrp	AGAGTTCTCAGGTCTAAGTCT	CTTGAAGAAGCGGCAGTAGCACGT	210
Cart	AGAAGAAGTACGGCCAAGTCC	CACACAGCTTCCCGATCC	84
Gdi	CCGCACAAGGCAAATACATC	GACTCTCTGAACCGTCATCAA	210
Leptin	TTCACACACGCAGTCGGTAT	AGGTCTCGCAGGTTCTCCAG	186
Npy	TGGACTGACCCTCGCTCTAT	GTGTCTCAGGGCTGGATCTC	188
Obrb	AGCCAAACAAAAGCACCATT	TCCTGAGCCATCCAGTCTCT	174
Pomc	CCTGTGAAGGTGTACCCCAATGTC	CACGTTCTTGATGATGGCGTTC	266
Socs-3	ACTGAGCCGACCTCTCTCCT	CCCCTCTGACCCTTTCTTTG	172

Abbreviations: Agrp, Agouti-related peptide; Cart, cocaine- and amphetamine-regulated transcript; Gdi, rho gdp dissociation inhibitor alpha; Npy, neuropeptide Y; Obrb, long-form leptin receptor; Pomc, proopiomelanocortin; Socs-3, suppressor of cytokine signalling-3. Thermal cycling conditions for all genes, with the exception of leptin were as follows: 10 min at 95°C followed by a total of 40 two-temperature cycles (15 s at 95°C and 1 min at 60°C). For leptin, conditions were as follows: 7 min at 95°C followed by a total of 50 three-temperature cycles (15 s at 95°C, 20 s at 60°C and 40 s at 72°C).

Morphometric and immunohistochemical analysis

In the fixed brains a coronal block containing the hypothalamus was cut, dehydrated in graded series of ethanol, cleared in xylene and embedded in paraffin. Coronal sections (5 μm thick) from the hypothalamus were cut using a microtome and mounted on Super-Frost/Plus slides.

Immunohistochemical demonstration of NPY in ARC and PVN was performed with the avidin-biotin peroxidise (ABC) method (Paxinos and Watson 1998; Hsu et al. 1981). Sections were incubated sequentially at room temperature in the following solutions: 0.3% hydrogen

peroxide in methanol for 10 min to block endogenous peroxidase; Citrate-based solution (pH 6) in microwave oven for 15 min and 20 min on ice for antigen retrieval; 2% goat normal serum in phosphate buffered saline (PBS) (pH 7.4-7.6) 0.1% Triton X-100 for 20 min to reduce non-specific background staining prior to incubation with primary antibody (polyclonal anti-NPY antibody produced in rabbit, N9528, Sigma-Aldrich, 1:800 in PBS 0.1% Triton X-100 with 1% BSA for 1 h and 15 min at 37 C); biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA) 1:200 in PBS 0.1% Triton X-100 with 1% BSA for 1 h at room temperature; peroxidase-labeled ABC reagent (Vectastain ABC kit, Vector) in PBS for 30 min at room temperature and Fast 3,3-diaminobezidine tablet, DAB (Sigma, St. Louis, MO,USA) in PBS 0.1% Triton X-100 for 3 min in dark room for enzymatic development of peroxidise. Subsequently, slides were washed with deionized water, dehydrated with increasing concentrations of ethanol and xylene, mounted with Eukitt (Panreac Quimica SA) and cover-slipped. Negative controls were performed by omission of primary antibody. Measurement of the number of immunoreactive NPY (NPY^+) neurons in ARC and the area occupied by NPY^+ fibers in PVN, were performed in 3 digitalized images/animal from the ARC (−2.3 to −3.3 mm posterior to Bregma) and from the PVN (−1.6 to −1.88 mm posterior to Bregma) according to published coordinates (Paxinos and Watson 1998) and with the help of hematoxylin/eosin staining.

The area occupied and the number of hematoxylin/eosin-stained cells were also measured. Capture of images (at a x10 magnification) and analysis were performed using AxioVision40V 4.6.3.0. Software (Carl Zeiss, Imaging Solutios GmbH, Germany). Image analysis from all groups was examined by two independent researchers in a blind fashion.

Statistical analysis

Data are reported as mean ± standard error (SEM). Two-way ANOVA with sex and group factors showed that male and female pups respond in a different manner to the treatments used. Therefore, one-way ANOVA was performed separately for each sex, to study individual differences between groups (controls, CR, CR-Leptin), followed by least significance difference (LSD) *post hoc* test. The data were confirmed for equality of variances by Levene's test ($p < 0.05$). Single comparisons between groups were assessed by Student's *t* test. Threshold of significance was set at $p < 0.05$. All the analyses were performed with SPSS Statistics 19.0 (SPSS, Chicago, IL).

Results

Body weight gain and energy intake in dams

Body weight gain and cumulative energy intake of dams during different periods of gestation and lactation are summarized in Table 2. 20% food restriction conducted at the beginning of gestation (day 1 to 12) resulted in lower weight gain of CR-dams and, consequently, these animals showed lower body weight at the end of the restriction period (day 12) with respect to their controls (controls-dams: 286 ± 3 g and CR-dams: 251 ± 3 g) ($p < 0.05$; Student's *t* test). During the second part of gestation (day 13 to 20), when all dams were allowed to eat *ad libitum*, CR-dams gradually regained body weight of controls, but at day 20 of gestation they still showed a slight tendency to lower body weight compared to their controls (controls-dams: 359 ± 7 g and CR-dams: 339 ± 8 g) ($p = 0.09$; Student's *t* test).

During the lactation period, although both groups of dams consumed a similar amount of food, CR-dams gained significantly more body weight than control-dams. Consequently, at the end of lactation (day 20) body weight of CR-dams achieved the level of control dams (controls-dams: 291 ± 5 g and CR-dams: 288 ± 4 g).

Table 2. Body weight gain and cumulative energy intake during different periods of pregnancy and lactation of dams with free access to standard chow diet (Control-dams) or subjected to 20% calorie restricted diet during the first 12 days of pregnancy (CR-dams).

		Control-dams	CR-dams
Pregnancy 1 - 12 days	Body weight gain (g)	41.0 ± 2.5	11.7 ± 2.1 *
	Cumulative energy intake (kcal)	724 ± 64	561 ± 7 *
Pregnancy 13 - 20 days	Body weight gain (g)	70.2 ± 4.7	86.0 ± 5.5
	Cumulative energy intake (kcal)	534 ± 45	604 ± 34
Lactation 1 - 20 days	Body weight gain (g)	22.4 ± 1.6	33.1 ± 3.9 *
	Cumulative energy intake (kcal)	3449 ± 77	3348 ± 78

Body weight gain was calculated as the increase of body weight from initial to final weight for each period, and is expressed in grams. Cumulative energy intake during different periods of gestation and lactation was calculated by adding the total amount of food consumed and multiplying by energy density of the diet (3.3 kcal/g), and is expressed in kilocalories. Results are expressed as the mean ± S.E.M. of 7-10 dams per group. Statistics: *, CR-dams different from Control-dams ($p < 0.05$; Student's *t*-test).

Energy intake, anthropometric measurements, circulating parameters and gastric leptin in the offspring

Data related with energy intake, body weight, body fat and body length measurements, circulating parameters and gastric leptin in the offspring at birth and/or after weaning are summarized in Figures 1A and B. Maternal caloric restriction during gestation did not affect body weight at birth. However, during the suckling period, CR pups gained less weight and exhibited lower body weight that their controls from postnatal day 6 onwards ($p < 0.05$; LSD *post hoc* one-way ANOVA test). Cumulative energy intake after weaning, from day 21 to day 25, was lower in CR animals with respect to controls ($p < 0.05$; LSD *post hoc* one-way ANOVA test), although no differences were found when this was referred to body weight.

Leptin treatment throughout lactation had no demonstrable effects on body weight of suckling pups, or in post-weaned animals at this juvenile age. However, post-weaned CR-Leptin females showed cumulative food intake slightly higher to that of CR animals, but not different to that of controls ($p < 0.05$; LSD *post hoc* one-way ANOVA test). At any rate, no differences were found when food intake was referred to body weight.

In addition, CR males, but not females, had shorter body length than their control counterparts, and both CR males and females exhibited lower body fat content than controls, expressed in absolute values, as well as in relative values referred to body weight ($p < 0.05$; LSD *post hoc* one-way ANOVA test). In turn, leptin treatment throughout lactation had no demonstrable effects on the mentioned parameters ($p < 0.05$; LSD *post hoc* one-way ANOVA test).

Analyses of circulating levels of glucose and insulin at postnatal day 25 showed no statistical differences between groups either due to caloric restriction during gestation or due to leptin treatment throughout lactation. However, in males, but not in females, maternal caloric restriction during gestation led to a significant reduction of leptin levels in plasma, which became partially reverted to the levels similar to controls due to leptin treatment throughout lactation ($p < 0.05$; LSD *post hoc* one-way ANOVA test). Similar trend to that of circulating leptin was found regarding leptin concentration in the stomach ($p < 0.05$; LSD post hoc one-way ANOVA test). However no significant changes between groups were found concerning gastric leptin mRNA levels.

A

	Males			Females		
	Control	**CR**	**CR-Leptin**	**Control**	**CR**	**CR-Leptin**
Cumulative energy intake (days 21-25)						
kcal	27.2 ± 0.5 **a**	25.4 ± 0.6 **b**	24.9 ± 0.7 **b**	26.1 ± 0.3 **a**	24.7 ± 0.6 **b**	25.0 ± 0.7 **a,b**
kcal/g body weight)	0.50 ± 0.01	0.51 ± 0.01	0.49 ± 0.01	0.51 ± 0.01	0.51 ± 0.01	0.51 ± 0.01
Anthropometric measurements						
Body weight, day 1 (g)	6.57 ± 0.18	6.37 ± 0.11	6.45 ± 0.12	6.38 ± 0.08	6.15 ± 0.13	6.18 ± 0.12
Body weigh,t day 21 (g)	47.0 ± 0.9 **a**	43.2 ± 0.9 **b**	43.9 ± 0.9 **b**	45.4 ± 0.6 **a**	42.2 ± 0.8 **b**	42.8 ± 0.8 **b**
Body weight, day 25 (g)	65.5 ± 1.1 **a**	59.6 ± 1.2 **b**	60.2 ± 1.2 **b**	61.7 ± 0.7 **a**	58.5 ± 1.2 **b**	57.0 ± 1.0 **b**
Body length, day 25 (cm)	12.4 ± 0.1 **a**	12.1 ±0.1 **b**	12.1 ± 0.1 **b**	11.9 ±0.1	11.8 ± 0.1	11.8 ± 0.1
Body fat, day 25 (g)	7.37 ± 0.28 **a**	5.56 ± 0.20 **b**	5.87 ± 0.22 **b**	6.93 ± 0.13 **a**	5.51 ± 0.18 **b**	5.68 ± 0.22 **b**
(%)	12.4 ± 0.3 **a**	10.1 ± 0.2 **b**	10.5 ± 0.2 **b**	12.2 ± 0.2 **a**	10.5 ± 0.2 **b**	10.5 ± 0.2 **b**
Circulating parameters (day 25)						
Glucose (mg/dL)	148 ± 4	145 ± 2	138 ± 4	149 ± 4	148 ± 5	142 ± 4
Insulin (ng/L)	170 ± 24	204 ± 33	221 ± 59	248 ± 41	240 ± 49	163 ± 52
Leptin (ng/L)	2206 ± 170 **a**	1798 ± 121 **b**	2042 ± 45 **a,b**	1689 ± 241	1461 ± 145	1414 ± 129
Stomach (day 25)						
Leptin (pg/g)	1544 ± 97 **a**	1230 ± 94 **b**	1309 ± 81 **a,b**	1515 ± 88	1705 ± 109	1445 ± 180
Leptin mRNA levels (%)	100 ± 13	81.3 ± 6.5	99.1 ± 9.5	89.1 ± 6.0	80.4 ± 7.6	95.9 ± 8.8

B

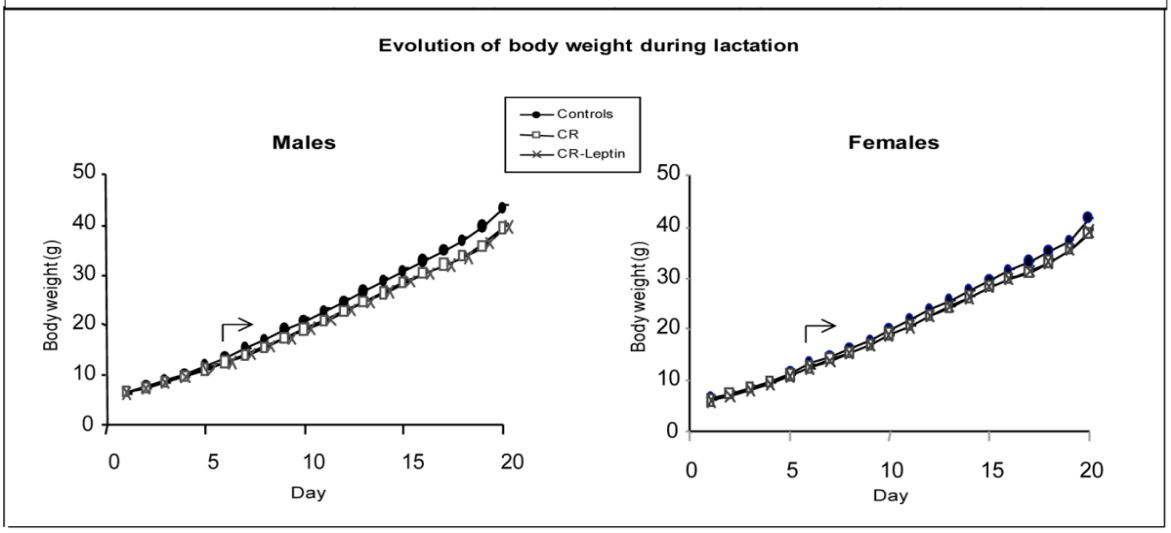

Figure 1. Offspring parameters. A. Energy intake, anthropometric measurements, circulating parameters and leptin mRNA and protein levels in stomach in the offspring of rats with free access to standard chow diet (control), the offspring of 20% calorie restricted dams during the first 12 days of pregnancy (CR), and CR rats daily supplemented with physiological doses of leptin throughout lactation (CR-Leptin). Cumulative energy intake (from postnatal day 21 to 25) was expressed in kcal, and also referred to body weight and expressed in kcal/g. Body weight was measured on postnatal days 1, 21 and 25. The other parameters were determined on day 25. Leptin mRNA levels in stomach were measured by qRT-PCR and expressed as a percentage of the value of control male rats. Leptin levels in stomach were quantified by ELISA and expressed in pg/g tissue. Data are mean ± S.E.M. For cumulative food intake, body weight at different days, body length and body fat content, n = 16-17; for mRNA analysis, n = 10-11; for circulating parameters, n = 6-8. Each group is made up of animals coming from at least six different litters. Statistics: in case of interaction within each sex, data not sharing a common letter (a and b) are significantly different (a≠b) ($p < 0.05$; LSD *post hoc* one-way ANOVA test). B. Evolution of body weight during lactation. The arrow indicates the starting point of significant effects of maternal caloric restriction during gestation on body weight in male and female offspring (CR≠Controls; CR-Leptin≠Controls; $p < 0.05$; LSD *post hoc* one-way ANOVA test).

Morphometry and immunohistochemistry of the ARC and PVN hypothalamic nuclei in the offspring

Morphometric (Figure 2) studies were performed in ARC and PVN hypothalamic nuclei. Analysis of ARC (Figure 2; left panels) showed that maternal caloric restriction during gestation produced an increase in the total number of hematoxylin/eosin-stained cells in the ARC of CR females compared to their controls ($p < 0.05$; LSD *post hoc* one-way ANOVA test), but a slight trend toward a decrease in CR males, compared to their controls. Leptin treatment throughout lactation reversed the effects observed in CR females diminishing significantly the total number of cells in ARC relative to their controls ($p < 0.05$; LSD *post hoc* one-way ANOVA test), as well as leading to the augmentation of their quantity in males (CR-Leptin *vs* CR males; $p < 0.05$; Student's *t*-test). A similar tendency was also observed by analyzing cell density. CR-Leptin animals showed the same levels as controls, but different compared to the CR groups ($p < 0.05$; LSD *post hoc* one-way ANOVA test). No differences were found concerning the area of ARC between experimental groups.

Morphometric studies in PVN (Figure 2; right panels), in turn, demonstrated that gestational caloric restriction also affected the development of this nucleus, by reducing the total number of cells in males, as well as by increasing their density in females in comparison to their control groups ($p < 0.05$; LSD *post hoc* one-way ANOVA test). These structural changes in PVN were restored in CR-Leptin animals ($p < 0.05$; LSD *post hoc* one-way ANOVA test).

In compliance with our previously published results (Garcia et al. 2010), immunohistochemical analysis of NPY^+ cells (Figure 3) showed that CR males, but not females, presented a significantly lower number of NPY^+ cells in the ARC nucleus, with respect to their controls ($p < 0.05$; LSD *post hoc* one-way ANOVA test). The observed diminishment in CR males was partially restored in CR-Leptin males, reaching levels not different to those of controls ($p < 0.05$; LSD *post hoc* one-way ANOVA test). With respect to the PVN (Figure 4), no significant differences were found in the area of NPY^+ fibers within experimental groups. However, when this value was expressed relative to the total area of the PVN, both male and female CR animals presented a higher % of NPY^+ fiber area. Leptin supplementation throughout lactation partially normalized these values to those observed in controls ($p < 0.05$; LSD *post hoc* one-way ANOVA test).

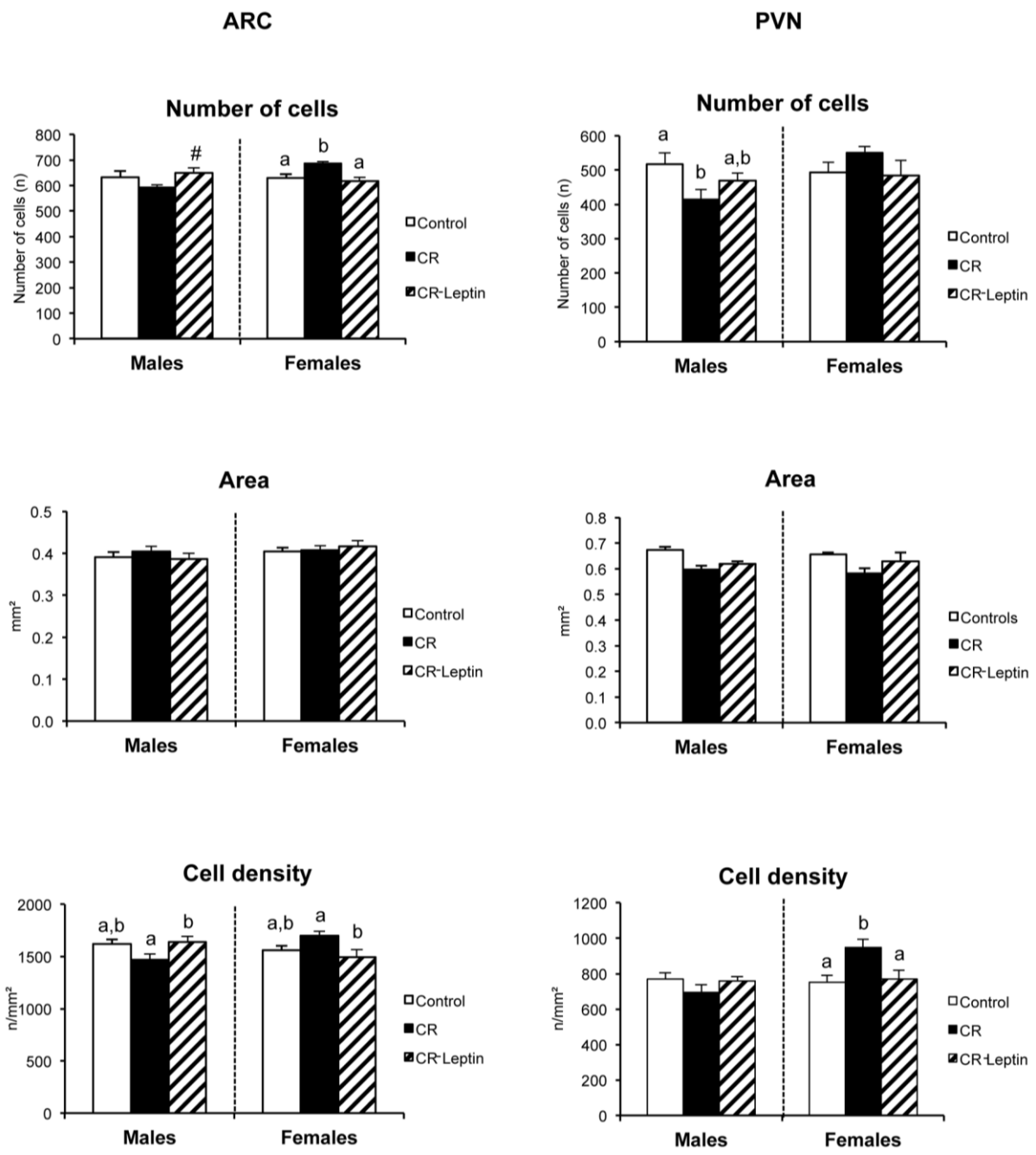

Figure 2. Morphometry of the ARC and PVN hypothalamic nuclei in the offspring. Total number of cells, area and cell density in the arcuate nucleus (ARC) (left panels) and total number of cells, area, and cell density in the paraventicular nucleus (PVN) (right panels) of the hypothalamus of 25-day-old male and female offspring of dams with free access to standard chow diet (controls), the offspring of 20% calorie restricted dams during the first 12 days of pregnancy (CR), and CR rats daily supplemented with physiological doses of leptin throughout lactation (CR-Leptin). The number of cells was determined in hematoxylin/eosin-stained section of the hypothalamus and the area was counted using computerized image analysis software. Neuronal density was calculated dividing total number of cells in nuclei per surface of nucleic area and is expressed as number of cells per square millimeter. Data are mean ± S.E.M. (n = 6-8, coming from at least six different litters). Statistics: $^{\#}$, CR-Leptin different from CR group ($p < 0.05$; Student's *t*-test); in case of interaction within each sex, bars not sharing a common letter (a and b) are significantly different ($a \neq b$) ($p < 0.05$; LSD *post hoc* one-way ANOVA test).

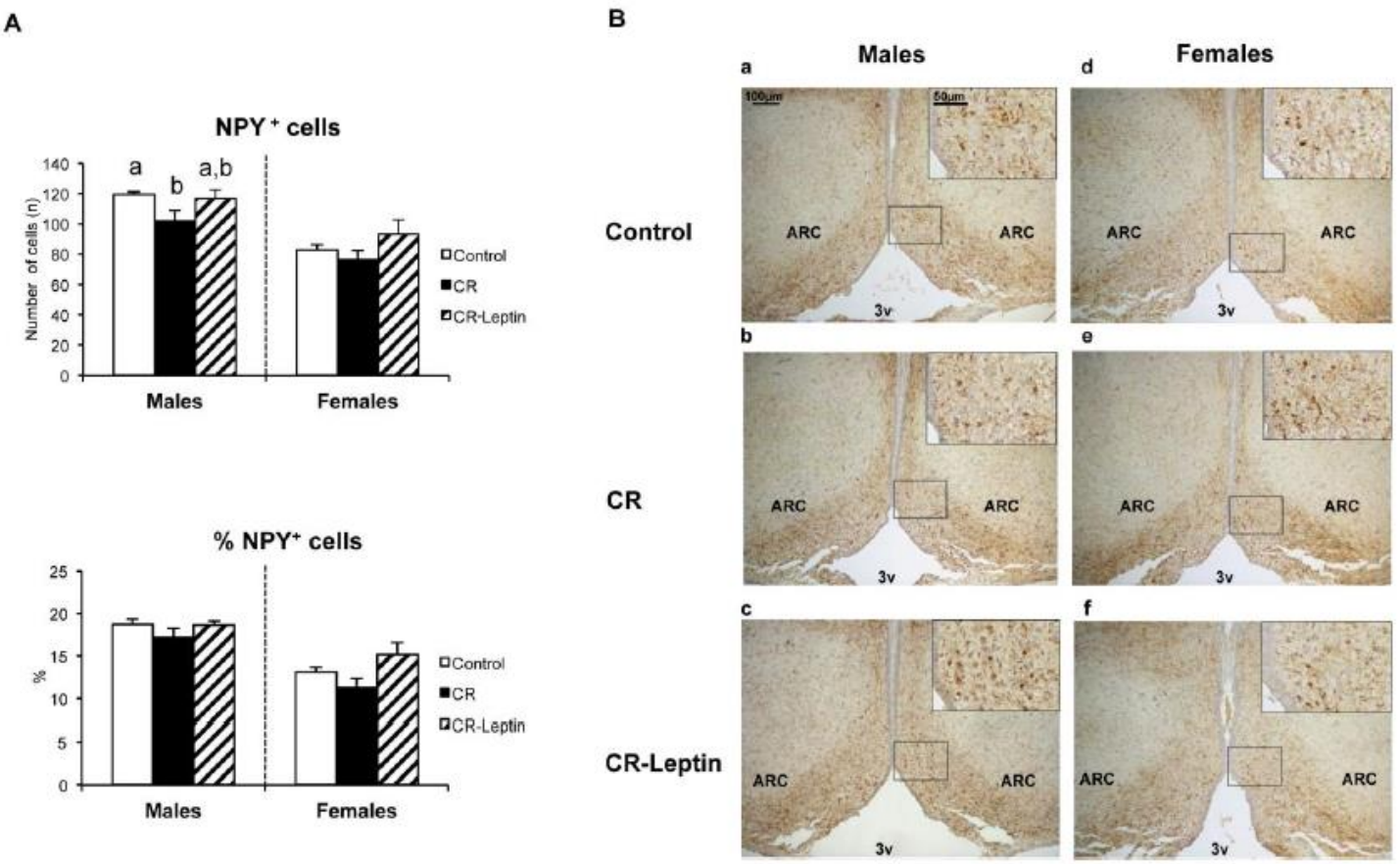

Figure 3. Immunohistochemistry of the ARC hypothalamic nucleus in the offspring. A. Number of NPY positive (NPY^+) cells and percentage of NPY^+ cells referred to the total number of cells in the arcuate nucleus (ARC) in the hypothalamus of 25-day-old male and female offspring of dams with free access to standard chow diet (controls), the offspring of 20% calorie restricted dams during the first 12 days of pregnancy (CR), and CR rats daily supplemented with physiological doses of leptin throughout lactation (CR-Leptin). Number of NPY^+ cells in the ARC was counted using computerized image analysis software, following immunostaining. Data are mean ± S.E.M. (n = 6-8, coming from at least six different litters). Statistics: in case of interaction within each sex, bars not sharing a common letter (a and b) are significantly different ($a \neq b$) ($p < 0.05$; LSD *post hoc* one-way ANOVA test). B. Representative brain sections immunostained for NPY in the arcuate hypothalamic nucleus of the offspring of 25-day-old male (a) and female (d) offspring of dams with free access to standard chow diet (controls), male (b) and female (e) offspring of 20% calorie restricted dams during the first 12 days of pregnancy (CR), and male (c) and female (f) CR rats daily supplemented with physiological doses of leptin throughout lactation (CR-Leptin). Insets: enlargement of the corresponding framed areas. Abbreviations: ARC, arcuate nucleus; 3v, third ventricle. Scale bar: 100 µm and 50 µm for insets.

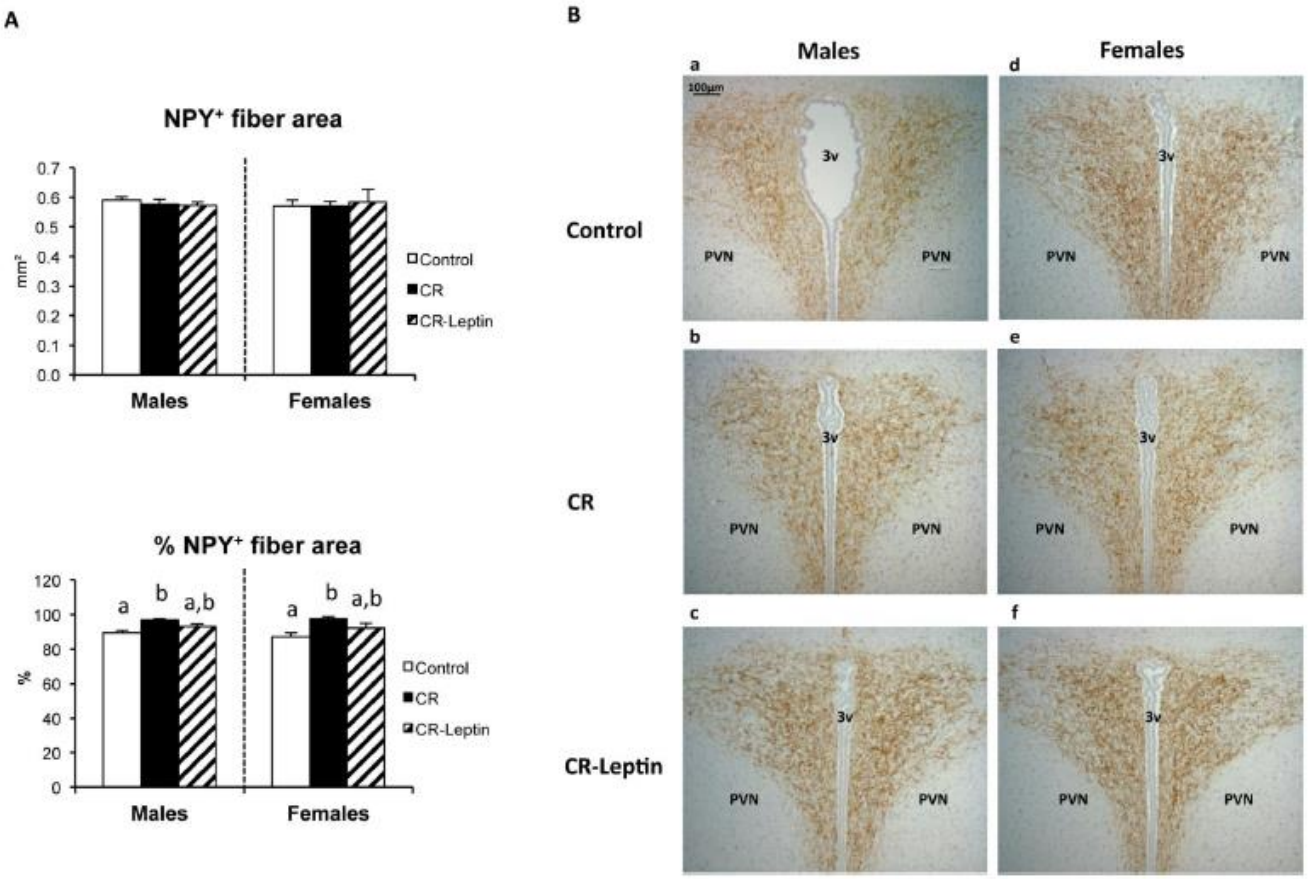

Figure 4. Immunohistochemistry of the PVN hypothalamic nucleus in the offspring. Number of NPY^+ fiber area and percentage of NPY^+ fiber area referred to the total area of the paraventicular nucleus (PVN) in the hypothalamus of 25-day-old male and female offspring of dams with free access to standard chow diet (controls), the offspring of 20% calorie restricted dams during the first 12 days of pregnancy (CR), and CR rats daily supplemented with physiological doses of leptin throughout lactation (CR-Leptin). The area of NPY^+ fibres (expressed in square millimeter) in PVN was estimated using computerized image analysis software, following immunostaining. Data are mean ± S.E.M. (n = 6-8, coming from at least six different litters). Statistics: in case of interaction within each sex, bars not sharing a common letter (a and b) are significantly different (a≠b) ($p < 0.05$; LSD *post hoc* one-way ANOVA test). B. Representative brain sections immunostained for NPY in the paraventicular hypothalamic nucleus of the offspring of 25-day-old male (a) and female (d) offspring of dams with free access to standard chow diet (controls), male (b) and female (e) offspring of 20% calorie restricted dams during the first 12 days of pregnancy (CR), and male (c) and female (f) CR rats daily supplemented with physiological doses of leptin throughout lactation (CR-Leptin). Abbreviations: PVN, paraventicular nucleus; 3v, third ventricle. Scale bar: 100 µm.

mRNA expression levels of Agrp, Cart, Npy, Obrb, Pomc and Socs-3 in hypothalamus in the offspring

To ascertain whether the apparent changes in hypothalamic structure between groups affected its function, the expression levels of selected hypothalamic genes involved in the control of food intake and energy balance were determined (Figure 5). No statistical differences were observed in the expression levels of InsR either due to gestational caloric restriction or due to leptin treatment throughout lactation (data not shown). Gestational caloric restriction did not affect the expression levels of ObRb either, however, leptin treatment throughout lactation resulted in overexpression of this gene in males, but not in females, with respect to both control and CR rats ($p < 0.05$; LSD *post hoc* one-way ANOVA test). Regarding SOCS-3 mRNA expression levels were significantly altered in CR animals, with different expression patterns between sexes. In comparison to controls, CR male animals exhibited lower SOCS-3 mRNA levels, whereas in CR females its mRNA levels were increased; interestingly, leptin treatment throughout lactation reversed these effects, resulting in a partial increase of SOCS-3 expression levels in males and a significant downregulation in females ($p < 0.05$; LSD *post hoc* one-way ANOVA test). In agreement with our previous study in another cohort of animals (Garcia et al. 2010), gestational caloric restriction led to lower expression levels of the orexigenic neuropeptide NPY; however, in the present study, the mentioned effect was observed in males, but not in females ($p < 0.05$; LSD *post hoc* one-way ANOVA test). In turn, leptin treatment throughout lactation brought about a partial reversion of mRNA levels of NPY in CR-Leptin group of males, which exhibited 16% rise in its expression levels compared to their CR counterparts. No statistical differences between groups were observed in the expression levels of the other orexigenic neuropeptide, AgRP. Concerning anorexigenic neuropeptides, no differences in POMC expression levels were displayed between groups, either due to gestational caloric restriction or due to leptin treatment throughout lactation. However, single comparison between groups revealed that females in CR-Leptin group exhibited a rise in POMC expression levels with respect to female offspring from normally nourished mothers (CR-Leptin *vs* Control females) ($p < 0.05$; Student's *t*-test). In accordance with our previous study in another cohort of animals (Garcia et al. 2010), gestational caloric restriction resulted in lower expression levels of another anorexigenic neuropeptide, CART; however, in this study the effect was observed in males, but not in females ($p < 0.05$; LSD *post hoc* one-way ANOVA test). In turn, leptin treatment throughout

lactation brought about a mild reversion of CART mRNA levels, since males in the CR-Leptin group exhibited 6% rise in its expression levels compared with males in the CR group.

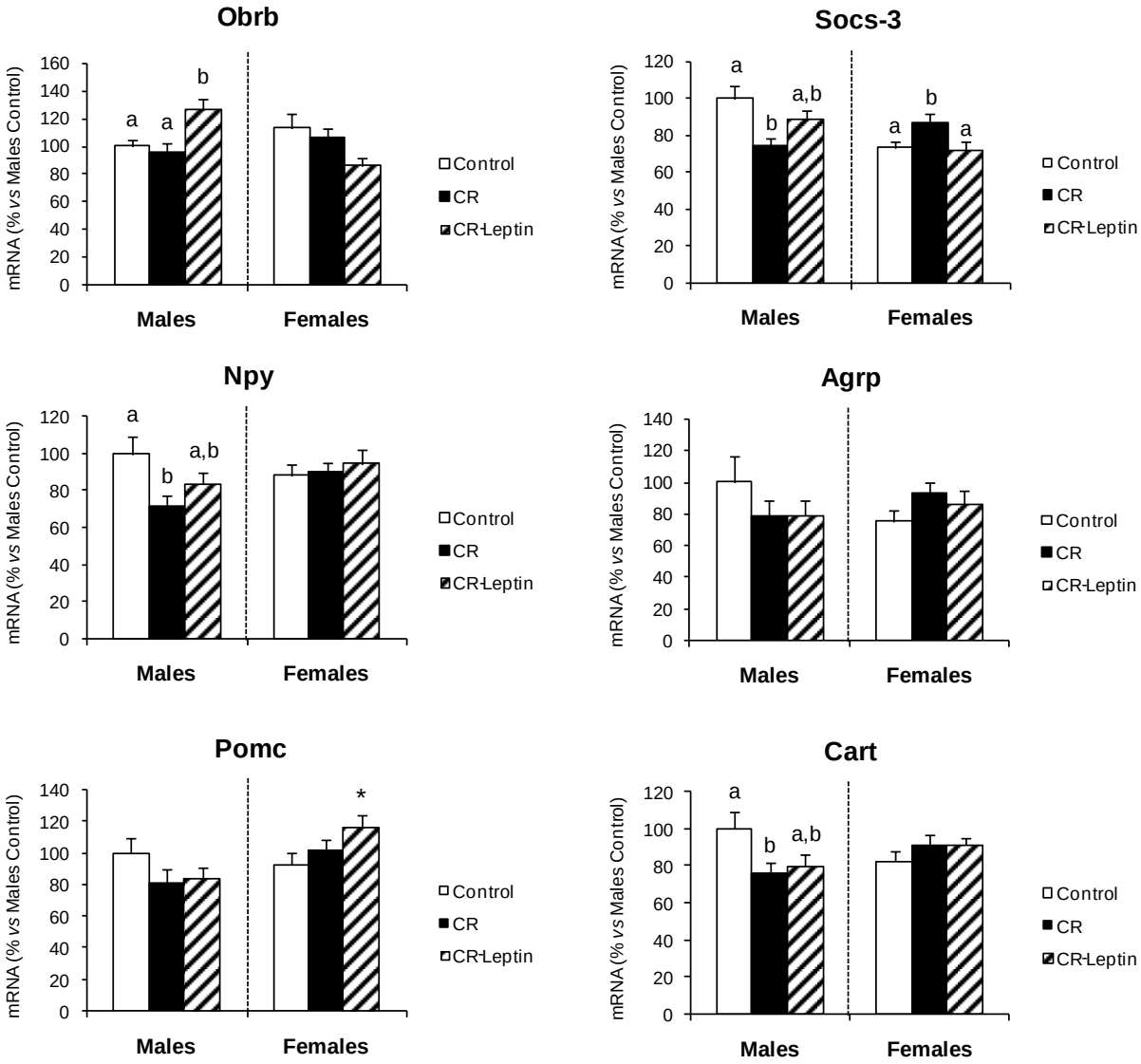

Figure 5. mRNA levels of Agrp, Cart, Npy, Obrb, Pomc and Socs-3 in hypothalamus in the offspring. Hypothalamic expression levels of Agrp, Cart, Npy, Obrb, Pomc and Socs-3 in 25-day-old male and female offspring of dams with free access to standard chow diet (controls), the offspring of 20% calorie restricted dams during the first 12 days of pregnancy (CR), and CR rats daily supplemented with physiological doses of leptin throughout lactation (CR-Leptin). mRNA levels were measured by qRT-PCR and expressed as a percentage of the value of control male rats. Data are mean ± S.E.M. (n = 10-11, coming from at least six different litters). Statistics: *, CR different from control group ($p < 0.05$; Student's *t*-test); in case of interaction within each sex, bars not sharing a common letter (a and b) are significantly different (a≠b) ($p < 0.05$; LSD *post hoc* one-way ANOVA test).

Discussion

We have previously shown that neonate male rats born to perinatally adequately nourished dams orally treated with physiological amounts of leptin throughout the suckling period are protected against the development of obesity and metabolic dysfunction in adulthood (Pico et al. 2007; Sanchez et al. 2008). Given that the hypothalamic circuitry that programs the capacity to regulate energy homeostasis becomes fully developed during a critical neonatal period, and is particularly susceptible to permanent programming by the early nutritional and hormonal environment (Bouret and Simerly 2006; Bouret et al. 2004; Spencer 2012; Garcia et al. 2010), here, we investigated the capacity of supplementation with physiological doses of leptin throughout lactation to reverse prior developmental malprogramming induced by maternal undernutrition during gestation. We used a rat model of 20% moderate maternal caloric restriction during the first part of pregnancy. This prenatal condition has been previously described to perturb offspring hypothalamic ARC structure and function (Garcia et al. 2010) and cause dysregulation of appetite in adulthood (Palou et al. 2012). This condition was also associated with disruption in the neonatal leptin surge (Palou et al. 2012). Interestingly, it was also found in these previous studies that although both genders showed hyperphagia, only males displayed higher body weight in adulthood, particularly under a high-fat diet (Palou et al. 2010a; Palou et al. 2012).

In the present study, the overall effects of gestational calorie restriction on neuroanatomic and metabolic health outcomes of juvenile rats, particularly in male offspring, were broadly consistent with previously reported results (Garcia et al. 2010). However, although in our previous study (Garcia et al. 2010) dams restricted calorically showed similar body weight to their controls at the end of the pregnancy, in the present study they exhibited a relatively slower body weight recuperation rate, and despite being fed *ad libitum* with a balanced diet after the restriction period, they managed to completely recover the previous body weight loss only toward the end of the lactation period. We also found here that undernutrition conditions in dams during gestation affected anthropometric parameters of the offspring. Gestational calorie restriction induced slowdown of growth in neonates, which resulted in lower body weight from postnatal day 6, as well as lower body fat mass and length (the latter only in males) compared to their controls (measured at weaning). In addition, food intake in post-weaned rats, from day 21 to day 25, was significantly lower in CR animals compared with controls. Oral supplementation with physiological doses of leptin throughout lactation to

the offspring of undernourished dams did not show any apparent effect on the growth of pups during this period. In rats born from adequately nourished dams during gestation, oral supplementation with the same doses of leptin during lactation, as used here, was previously shown to inhibit food intake, however no effect was either found on body weight gain during treatment (Pico et al. 2007; Sanchez et al. 2008). Vickers *et al.* observed that daily leptin subcutaneous injection of pharmacological doses (2.5 µg/g/d) from postnatal day 3 to13 resulted in a slowdown of body weight gain during treatment in the offspring of prenatal 30% calorie-restricted dams, which was suggested to be associated with direct effects of leptin on energy expenditure (Vickers et al. 2005; Vickers et al. 2008). The apparent differences in the effects of leptin during lactation concerning energy balance regulation during the treatment period may be attributed to the administration technique, doses, as well as to the nutritional status of pups.

Leptin has been shown to play an essential role during critical windows of development in the pathogenesis of programming related disorders (Palou and Pico 2009; Pico et al. 2011). Maintaining a critical leptin level during development may allow the normal maturation of tissues and pathways involved in metabolic homeostasis (Vickers 2007)**.** In normally developing rodents there is an increase in circulating levels of leptin between postnatal days 4-16, the so-called leptin surge (Ahima et al. 1998). Disruption in leptin surge during perinatal life in rodents has been demonstrated to have lasting consequences by altering the capacity to respond to leptin in adulthood, thus predisposing animals to obesity (Attig et al. 2008; Pico et al. 2011). In fact, we and others have reported that perinatal caloric restriction triggers a reduction or even absence of neonatal leptin surge (Palou et al. 2012; Delahaye et al. 2008), leading to severe alterations in the control of energy balance in adulthood. As a matter of fact, in a previous study applying the same model of gestational caloric restriction, but conducted in another cohort of animals (Palou et al. 2012), we showed an absence of the leptin surge, occurring in their controls at postnatal day 9. Here, although we did not measure leptin levels during the suckling period, we detected that 25-day-old CR male animals displayed lower circulating leptin levels than their controls. In turn, leptin supplementation throughout lactation normalized their levels to those of controls. This leads us to speculate that the leptin surge was also ameliorated in those animals, and this might be the cue, which brings reversion of altered programming cascade. Similarly, Vickers *et al.* reported that daily leptin treatment from postnatal day 3 to 13 normalized fasting plasma leptin concentration in female adult offspring of calorie-restricted dams during pregnancy (Vickers et al. 2005);

however, data confirming this normalization during the neonatal and juvenile period were not available in this study.

The origin of leptin allowing the normalization of its circulating levels in 25-day-old CR-Leptin male animals to those of controls is not known. It does not seem to be directly caused by oral leptin supplementation, since this treatment lasted only until day 21. Moreover, leptin absorption by the stomach in rat pups has been described to be more significant during the first part of the suckling period, but decreases when animals start to eat a solid diet (Oliver et al. 2002). To ascertain which endogenous supplier was allowing the normalization of circulating leptin concentration in these animals, leptin production was measured in the gonadal adipose tissue depot and in the stomach. Results showed that adipose tissue is unlikely to be responsible because CR-Leptin animals showed no increased fat mass compared with CR animals, nor was higher leptin expression found in this tissue (data not shown). However, although leptin expression levels in the stomach were not significantly different between groups, CR males exhibited lower gastric leptin levels than controls, whereas levels were normalized in CR animals supplemented with leptin during the suckling period, a similar trend to that found for circulating levels. Therefore, it could be speculated that changes in leptin production by the stomach in male animals could contribute to changes in circulating leptin levels, particularly during this period when the transition from milk to solid food occurs and prior to a greater contribution of adipose tissue. It must be highlighted that the alteration in circulating or gastric leptin levels occurring in male offspring as a consequence of gestational maternal caloric restriction was not evident in female animals. No changes were found as a consequence of leptin treatment during lactation either. This sex-dependent difference in leptin profile as a consequence of maternal caloric restriction may account, at least in part, for the different sex-dependent outcomes of this condition in hypothalamus structure and function (described below and summarized in table 3) as well as in adult phenotype, as previously described (Palou et al. 2012).

In neonate rodents, the neuronal network responsible for food intake and energy balance regulation is progressively established during early postnatal life; in fact, elevated leptin levels during the critical window of development corresponds to the developmental activity of leptin on hypothalamic neuronal circuitry (Bouret and Simerly 2006). Morphometric analysis of the hypothalamus in this and other studies have evidenced that gestational caloric restriction affects hypothalamic structure (Delahaye et al. 2008; Ikenasio-Thorpe et al. 2007; Garcia et al. 2010). In turn, exogenous leptin administration at pharmacological doses

reverses some of the neuroanatomical defects associated with absence or alterations in neonatal leptin surge during the critical window of developmental plasticity (Bouret et al. 2004; Bouret and Simerly 2006). Interestingly, here we show that oral supplementation with physiological doses of leptin throughout lactation normalized the total number of cells and cell density in ARC and PVN in the offspring of caloric restricted dams during gestation. Notably, in females, gestational malprogramming did not reduce cell density in PVN, but triggered its increase. In turn, leptin treatment in CR females also brought about its correction. In addition, in agreement with our previous study (Garcia et al. 2010), we found that gestational caloric restriction led to a reduction of immunostained NPY^+ cells in ARC of CR males, but no changes were found in females. The decrease occurring in CR males was corrected by inducing leptin supplementation throughout lactation. Therefore, according to the neurotrophic action of leptin on the ARC, we could speculate that the restoration of the ARC structure could be a consequence, at least in males, of restored plasma leptin levels. Nevertheless, no obvious difference was observed in the innervations of the PVN by NPY-containing projections, either due to gestational caloric restriction or due to leptin treatment. A similar observation was reported by Delahaye *et al.*, showing that 50% perinatal caloric restriction did not trigger gross abnormalities in the hypothalamic NPY projections from ARC to PVN (Delahaye et al. 2008). However, both male and female CR animals presented a higher relative area of NPY^+ fibers in the PVN, referred to the total area of this nucleus; this could be tentatively related with a higher predisposition to hyperphagia, as previously described (Palou et al. 2012). Interestingly, leptin supplementation throughout lactation partially normalized this ratio to the control values, both in male and female animals.

The rationale for differences between sexes in the effects of moderate maternal caloric restriction during gestation on hypothalamus structure, particularly affecting total number and density of cells in the ARC and PVN, is not known, but may reflect the interaction between nutritional signals and hormones. As mentioned above, these sex-dependent outcomes may be potentially related with the different effects of this condition on circulating leptin levels, since only males were significantly affected. Notably, it must be pointed out that the detrimental consequences of this condition during prenatal life in adulthood have also been found to be more marked in males than in females (Jones and Friedman 1982; Jones et al. 1984; Palou et al. 2010a). These results strongly suggest the important role of leptin during a specific window of development to adequately match the physiology of the neonate to its future environment, as previously suggested (Djiane and Attig 2008).

Table 3. Summary of changes in hypothalamus structure and function.

		Males		Females	
		CR vs C	**CR-Leptin vs C**	**CR vs C**	**CR-Leptin vs C**
ARC	Number of cells	–	–	↑	–
	Area of cells	–	–	–	–
	Cell density	–	–	–	–
	Number of NPY^+ cells	↓	–	–	–
	% of NPY^+ cells	–	–	–	–
PVN	Number of cells	↓	–	–	–
	Area of cells	–	–	–	–
	Cell density	–	–	↑	–
	NPY^+ fiber area	–	–	–	–
	% of NPY^+ fiber area	↑	–	↑	–
Obrb mRNA levels		–	↑	–	–
Socs-3 mRNA levels		↓	–	↑	–
Npy mRNA levels		↓	–	–	–
Agrp mRNA levels		–	–	–	–
Pomc mRNA levels		–	–	–	↑
Cart mRNA levels		↓	–	–	–

Arrows (↑ or ↓) indicates significant changes (increase or decrease, respectively) occurring in the male and female offspring of 20% calorie restricted dams during the first 12 days of pregnancy (CR) vs controls and in CR rats daily supplemented with physiological doses of leptin throughout lactation (CR-Leptin) vs controls; – indicates no significant changes. Notably, changes occurring in CR animals versus controls were not present in CR-Leptin animals versus controls, which is indicative that oral leptin during lactation is able to revert, at least partly, most of the sex-dependent neuroanatomic consequences in the offspring caused by moderate maternal caloric restriction during gestation. See material and methods for statistical details.

We also show here that changes in hypothalamic structure in CR animals were associated with changes in hypothalamic function, affecting expression levels of neuropeptides and factors involved in the regulation of feeding behavior, such as Npy, Cart and Socs-3. Interestingly, oral administration of physiological doses of leptin throughout lactation corrected expression levels of these factors. These adjustments were dependent upon prior developmental programming. Concerning Npy, and similarly to our observation in NPY^+ immunopositive cells in ARC, the decreased hypothalamic Npy mRNA expression levels occurring in CR male pups were restored in leptin treated animals. Similar effects were observed concerning Cart mRNA expression levels. Leptin treatment throughout lactation also reversed programmed altered expression of Socs-3, with opposite patterns between sexes. The production of SOCS-3 is considered as a marker of functional activation of leptin receptor and intracellular signaling (Bjorbaek et al. 1998); hence, the restoration of higher

levels of Socs-3 mRNA in CR-Leptin males (similar to controls) could be indicative of a restoration of leptin signaling. However, it is difficult to interpret those changes occurring in females, which are in the opposite direction with respect to males, although they mirror the pattern of changes in total number of cells in ARC and cell density in PVN. On the other hand, although maternal caloric restriction did not influence anorexigenic neuropeptide Pomc mRNA levels, it is worth pointing out that CR-Leptin females exhibited relatively higher Pomc expression levels with respect to female offspring from normally nourished mothers, what may make them more predisposed for effective ways to control food intake in adulthood. Similarly, hypothalamic Obrb mRNA levels were not significantly affected by maternal caloric restriction during gestation, but CR-Leptin male pups presented higher mRNA expression levels of Obrb than control and CR animals. This suggests that these rats may be more responsive to leptin action, what might confer certain protection against obesity in adulthood. This has not been directly measured here but, leptin supplementation during the suckling period to the offspring of adequately nourished rats, with the same doses as in the present study, was described to improve leptin sensitivity in adulthood (Sanchez et al. 2008). Appetite-related neuropeptides, as well as Socs-3 and Obrb, have also been reported to be sensitive to leptin treatment in neonates (Proulx et al. 2002). However, in that study, offspring of adequately nourished dams were intraperitonealy treated with high doses of leptin, thus the interplay between developmental malprogramming and alterations in expression levels of those genes was not addressed.

In conclusion, we have evidenced that oral supplementation with physiological doses of leptin throughout lactation has the ability to reverse, at least partly, most of the sex-dependent neuroanatomic consequences in the offspring caused by moderate maternal caloric restriction during gestation. This is the first demonstration that a specific compound during lactation may reverse a detrimental trend for obesity acquired by poor nutrition during pregnancy. More concretely, these findings support the relevance of the intake of appropriate doses of leptin throughout lactation, which should be worth considering when searching for strategies to treat and/or prevent development of obesity and its related metabolic disorders starting in the early stages of life.

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MANUSCRIPT 4

Leptin supplementation in suckling rats recuperates altered white adipose tissue sympathetic innervation and function caused by maternal caloric restriction during gestation

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Title page

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Conflict of interest: A. Palou, C. Picó and J. Sánchez are authors of a patent held by the University of the Balearic Islands on the use of leptin as an essential nutrient during lactation. The name of the patent is "Use of leptin for the prevention of excess body weight and composition containing leptin" (WO 2006089987 A1) (priority data: 23 Feb 2005).

Abstract

Maternal calorie restriction during gestation in rats has been associated with altered WAT sympathetic innervation and function of the offspring. Here, we aimed to investigate whether oral supplementation during the suckling period with leptin (a protein naturally present in breast milk) may reverts the aforementioned adverse programming effects. Three groups of male and female rats were studied at the age of 25 days: the offspring of *ad libitum* fed dams (controls), the offspring of 20% calorie-restricted dams during the first part of pregnancy (CR), and CR rats supplemented with physiological doses of leptin throughout lactation (CR-Leptin). Tyrosine hydroxylase (TyrOH) levels and its immunoreactive area, and mRNA expression levels of lipid metabolism-related genes and of deiodinase iodothyronine type II (Dio2) were determined in inguinal WAT (iWAT). Triiodothyronine (T3) levels were determined in blood. In CR males, leptin treatment restored the decreased TyrOH levels and its immunoreactive area in iWAT, accompanied by partial normalization of the underexpressed genes related to lipolysis and fatty acid oxidation (adipose triglyceride lipase, hormone-sensitive lipase, carnitine palmitoyltransferase 1, and peroxisome proliferator activated receptor gamma coactivator 1 alpha in this tissue. Leptin treatment also reverted the decreased T3 plasma levels and lipoprotein lipase mRNA levels occurring in CR animals (males and females) and the decreased Dio2 mRNA levels in CR females. Thus, leptin supplementation orally throughout lactation reverts most of the malprogrammed effects on WAT structure and function induced by poor nutrition during pregnancy. These findings support the relevance of the intake of leptin, bearing clear characteristics of essential nutrient during lactation, and as a strategy to treat and/or prevent the programmed trend to obesity acquired by inadequate fetal nutrition.

Keywords: leptin treatment; gestational calorie-restriction; tyrosine hydroxylase; lipid metabolism; triiodothyronine

Introduction

Besides the known contributing factors to obesity, such as lifestyle and genetic components, it is becoming increasingly apparent that nutritional environment in early stages of development may permanently program future metabolic health and propensity to obesity development in adulthood (Spencer 2012; Pico et al. 2012). In this regard, maternal undernourishment during gestation has been associated with lasting detrimental effects on homeostatic control of energy balance and increased susceptibility to obesity in the offspring (Pico et al. 2012; Jones et al. 1984; Bellinger et al. 2006), particularly when exposed postnatally to a high-fat diet (Palou et al. 2010; Palou et al. 2012; Vickers et al. 2000; Thompson et al. 2007). Perturbations in the structure of key organs have been proposed as one of the potential mechanisms that may elucidate the link between maternal nutrition and adverse health outcomes in the offspring (Pico et al. 2012). In fact, in animal models of maternal undernutrition during critical developmental periods as gestation and lactation, alterations in the structures of the central nervous system (CNS) involved in the control of food intake and energy expenditure have been proposed to account for the impaired capacity to regulate energy homeostasis in adult offspring (Delahaye et al. 2008; Garcia et al. 2010). Notably, the development of the peripheral structures of the nervous system has also been described to be affected by nutritional disturbances during these critical periods, such as sympathetic innervations of gut (Santer and Conboy 1990; Conboy et al. 1987), stomach (Garcia et al. 2013) and white (Garcia et al. 2011) and brown (Palou et al. 2014) adipose tissues. Concerning WAT, we have previously described that 20% maternal calorie restriction during gestation reduced sympathetic innervation of inguinal WAT (iWAT) in male offspring, which was accompanied by increased adiposity in adulthood (Garcia et al. 2011). Thus, perturbations in the structure of the sympathetic nervous system (SNS) were also proposed to account, at least in part, for lasting adverse effects of this perinatal condition on the control of energy metabolism.

Regarding obesity prevention, the role of leptin as an essential component during lactation for the programming of a lean phenotype, as well as a strategy for reversing metabolic disorders induced as a consequence of developmental malprogramming, has been addressed (Pico et al. 2011; Vickers and Sloboda 2012; Konieczna et al. 2013; Vickers et al. 2005; Vickers et al. 2008; Pico et al. 2012). The hormone leptin is mainly

produced by the adipose tissue, but is also naturally present in significant amounts in breast milk (Houseknecht et al. 1997; Casabiell et al. 1997), but not in infant formulas (O'Connor et al. 2003). Studies in infants have evidenced positive effects of breastfeeding upon formula feeding, since this has been associated with lowering the risk of childhood obesity (von Kries et al. 1999; Armstrong and Reilly 2002), among other positive effects. Moreover, a negative association has been found between milk-borne maternal leptin levels and body weight gain of infants (Miralles et al. 2006; Schuster et al. 2011). In experimental animal models, neonatal leptin treatment in rats has been found to prevent age-related body fat accumulation and other metabolic alterations associated with high-fat diet feeding (Pico et al. 2007; Sanchez et al. 2008; Priego et al. 2010). Other studies carried out in animal models of maternal undernutrition evidenced that exogenous leptin treatment to the offspring reverses postnatal sequelae induced by developmental programming, preventing the development of a programmed trend to obesity and other metabolic alterations in later life (Vickers et al. 2005; Vickers et al. 2008; Konieczna et al. 2013). Vickers et al. (Vickers et al. 2005; Vickers et al. 2008) showed that leptin injections into neonatal rats born to undernourished mothers normalized the altered phenotype in adulthood induced as consequence of developmental programming, including calorie intake, locomotor activity, body weight, fat mass, as well as insulin and leptin concentrations. Moreover, we have previously shown that daily administration of oral leptin at physiologic doses throughout suckling period reverted most of the developmentally programmed outcomes induced by moderate maternal calorie restriction during gestation on central structures of the nervous system involved in the control of food intake and energy expenditure (Konieczna et al. 2013). These findings suggest that these developmentally programmed alterations, which have generally been considered permanent, might be reversed to some extent.

Bearing in mind the importance of the SNS for the control of WAT metabolic function, we used the model of 20% mild/moderate maternal calorie restriction during gestation, which has been shown to be associated with reduced sympathetic innervation of iWAT, particularly in male pups, to investigate whether supplementation with physiological oral doses of leptin throughout the suckling period is able to reverse the malprogrammed effects in iWAT structure and function.

Materials and methods

Animals and Experimental Design

The study was conducted in male and female Wistar rats from 17 different litters following the animal protocol that has been previously described in detail (Konieczna et al. 2013). Briefly, animals were housed under standard conditions, that is, controlled temperature (22 °C), the normal 12-h light and 12-h dark cycle, free access to tap water and a standard laboratory rodent chow diet (3.3 kcal/g, with 8% calories from fat; Panlab, Barcelona, Spain), unless specified. Virgin female Wistar rats (body weight 217 g - 244 g) were mated with male rats (Charles River Laboratories, Barcelona, Spain). Day of conception (day 0 of pregnancy) was determined by examination of vaginal smears for the presence of sperm. The group of control dams (n=7 animals) was fed *ad libitum* with standard chow diet, and the group of calorie restricted dams (CR-dams) (n=10 animals) was supplied with 20% calorie restriction from day 1 to day 12 of gestation. After the calorie restriction period all dams were fed *ad libitum*.

On day 1 after delivery, excess pups in each litter were removed to keep 10 pups per dam. Pups of both sexes born from CR-dams were randomly assigned into two groups: CR and CR-Leptin. CR-Leptin animals were supplemented each day throughout lactation with an oral solution of recombinant murine leptin (PeproTech, London, UK) dissolved in water with the use of a pipette. The amount of leptin given to animals was progressively increased from 1 ng of leptin on day 1, to 43.8 ng of leptin on day 20 of life, as previously described (Pico et al. 2007). CR pups and the offspring of control dams (controls) received the same volume of the vehicle (water). Weaning was performed at the age of 21 days, and then, 35 pups from control group (18 males and 17 females), 34 from CR group (17 males and 17 females), and 33 from CR-Leptin group (17 males and 16 females) were housed in groups of two animals, and fed on a standard chow diet until their sacrifice at the age of 25 days, under fed conditions.

At sacrifice, some of the pups (n = 10–11, per group) were used for gene expression analysis and the others (n = 6-8, per group) to perform morphometric and immunohistochemical analysis. Animals used for the different analysis were from at least six different litters. Blood samples were collected (n = 6-8, per group) in heparinized containers, then centrifuged at 1000 x g for 10 min to obtain the plasma, and stored at −20 °C until analysis. For gene expression studies, the entire inguinal

depot of WAT (iWAT) was rapidly removed, frozen in liquid nitrogen and stored at −80 °C until RNA analysis. For morphometric and immunohistochemical analysis, iWAT samples were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4) at 4 °C for 24 h, then washed and stored in 0.1 M phosphate buffer (pH = 7.4) until posterior analysis.

The animal protocol followed in this study was reviewed and approved by the Bioethical Committee of the University of the Balearic Islands and guidelines for the use and care of laboratory animals of the University were followed.

Quantification of glucose, insulin, leptin and T3 concentration in plasma

Blood glucose concentration was measured by Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Peripheral hormones were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits: insulin (Mercodia AB, Uppsala, Sweden), leptin (R&D Systems, Minneapolis, MN, USA), and triiodothyronine (T3) (DRG Instruments GmbH, Marburg, Germany), according to the manufacturer's instructions.

Total RNA isolation from iWAT

Total RNA was extracted from iWAT of pups with use of NucleoSpin® TriPrep kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) following the manufacturer's instructions. RNA yield was quantified on the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies, Wilmington, DE, USA) and its integrity confirmed using 1% agarose gel electrophoresis.

Real-time quantitative RT-polymerase chain reaction (RT-qPCR) analysis

mRNA expression levels of adipose triglyceride lipase (Atgl); the muscle isoform of carnitine palmitoyltransferase 1 (Cpt1b); deiodinase iodothyronine type II (Dio2); hormone sensitive lipase (Lipe); lipoprotein lipase (Lpl) and peroxisome proliferator activated receptor gamma coactivator 1 alpha (Pgc1a) in iWAT were analyzed by RT-qPCR as previously described (Palou et al. 2012), using β-actin as endogenous control. Sequences of primers (Sigma, Madrid, Spain) were as follows: β-actin, forward: 5´-tacagcttcaccaccacagc-3´, reverse: 5´-tctccagggaggaagaggat-3´; Atgl, forward: 5´-tgtggcctcattcctcctac-3´, reverse: 5´-agccctgtttgcacatctct-3´; Cpt1b, forward: 5´-

gcagaaaccgaagaaaggaa-3´, reverse: 5´-ctggagaaagggagatacaagg-3´; Dio2, forward: 5´-ttctccaactgcctcttcct-3´, reverse: 5´-caggtcgctgaaccaaagt-3´; Lipe, forward: 5´-tcacgctacataaaggctgct-3´, reverse: 5´-ccacccgtaaagagggaact-3´; Lpl, forward: 5´-tatggcacagtggctgaaag-3´, reverse, 5´-ctgaccagcggaagtaggag-3´; Pgc1a, forward: 5´-catttgatgcactgacagatgga-3´, reverse: 5´-ccgtcaggcatggaggaa-3´.

Western blot analysis of tyrosine hydoxylase (TyrOH) in iWAT

The amount of tyrosine hydroxylase (TyrOH) in iWAT of control, CR and CR-Leptin male and female rats at the age of 25 days was determined by Western blot. Tissue samples were homogenized at 4°C in 1:4 (w:v) of phosphate buffer saline (PBS) (137mM NaCl, 2.7mMKCl, 10mM Na_2HPO_4, adjusted pH 7.4) with protease inhibitors (10µl/ml PMSF, 1µl/ml leupeptin and 1µl/ml aprotinin). The homogenate was centrifuged at 7500*g* for 2 min at 4°C, and the supernatant was used for protein analysis. Total protein content was measured by the method of Bradford (Bradford 1976). For analysis, 100 µg of total protein was solubilized and boiled for 3 min in Laemmli sample buffer containing 5% 2-beta-mercaptoethanol. Then, total protein was fractionated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE; 12% polyacrylamide) and electrotransferred onto a nitrocellulose membrane (Bio-Rad, Madrid, Spain). After blocking, the membrane was incubated with the primary rabbit polyclonal anti-TyrOH (Santa Cruz Biotechnology, Inc., CA, USA) diluted 1:2000, and then with the infrared (IR)-dyed secondary anti-IgG antibody (LI-COR Biosciences, NE, USA) diluted 1:20,000. For IR detection, membranes were scanned in Odyssey Infrared Imaging System (LI-COR Biosciences, NE, USA), and the bands were quantified using the analysis software provided. The band intensity for TH protein was normalized to that of β-actin (mouse monoclonal anti-β-actin antibody, diluted 1:4000) (Cell Signaling Technology, Inc., USA).

Morphometric analysis of iWAT and immunohistochemical analysis of tyrosine hydoxylase (TyrOH) in iWAT

The fixed samples of iWAT were dehydrated in graded series of ethanol, cleared in xylene and embedded in paraffin. Sections of tissues (5 µm thick) were cut using a microtome and mounted on Super-Frost/Plus slides. The sections were stained with hematoxylin-eosin solution for 5 min and analyzed with the use of an optical

microscope equipped with a digital camera. Microscope images of the sections were captured at 10x magnification and analyses were performed using AxioVision40V 4.6.3.0. software (Carl Zeiss, Imaging Solutios). Images of adipocytes were obtained and their diameter was determined.

Sections were incubated sequentially at room temperature in the following solutions: 0.3% hydrogen peroxide in distilled water for 10 min to block endogenous peroxidase; 2% goat normal serum in phosphate buffered saline (PBS) (pH 7.4-7.6) for 20 min to reduce non-specific background staining prior to incubation with primary antibody (anti-TyrOH rabbit polyclonal antibody) (AB1542, Chemicon International) diluted 1:250 in PBS for 24-h at 4°C; biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA) 1:200 in PBS for 30 min at room temperature; peroxidase-labeled ABC reagent (Vectastain ABC kit, Vector) 1:100 in PBS for 30 min at room temperature and 3,3-diaminobezidine tablet, DAB (Sigma, St. Louis, MO,USA) in distilled water for 3 min in a dark room for enzymatic development of peroxidase. Subsequently, slides were washed with distilled water, dehydrated with increasing concentrations of ethanol and xylene, mounted with Eukitt (PanreacQuimica SA) and cover-slipped. Brown adipose tissue (BAT) sections were used as a positive control for TyrOH immunoreactivity.

Images were captured (at 10x magnification) and analyzed using AxioVision40V 4.6.3.0. software (Carl Zeiss, Imaging Solutios GmbH, Germany). For each animal, 14–20 inguinal images from light microscopy, coming from two nonconsecutive sections (7–10 fields per section), were chosen randomly and digitalized. The specific immunoreactive signal was recognized as the intensive brown color usually observed near arterioles and capillaries. The software was also used to measure the diameter of adipocytes in hematoxylin/eosin stained sections (different from those in which immunohistochemistry was performed). On one random field taken from each section, the diameter of all adipocytes was measured and averaged. Image analysis from all groups was examined in a blind fashion.

Statistical analysis

Data are reported as mean ± standard error (SEM). Multiple comparisons were assessed by two-way ANOVA (considering males and females) and by one-way ANOVA (separately for each sex) to study individual differences between groups (controls, CR,

CR-Leptin), and followed by least significance difference (LSD) *post hoc* test. The data were confirmed for equality of variances by Levene's test ($p< 0.05$). Single comparisons between groups were assessed by Student's *t* test. $P<0.05$ was the threshold of significance, unless stated. All the analyses were performed with SPSS Statistics 19.0 (SPSS, Chicago, IL).

Results

Phenotypic characteristics and blood parameters in the offspring

Detailed characterization of energy intake, body weight evolution and blood parameters of the same cohort of animals at the age of 25 days has been previously published (Konieczna et al. 2013). Some of these data are summarized here (Table 1). The weight and adipocyte diameter of iWAT and blood T3 levels are also included in the table. Maternal calorie restriction during gestation affected all of the weight-related and morphological traits studied in 25-day-old offspring. Specifically, CR male and female pups exhibited lower body weight and body fat content compared to their controls ($p<0.05$; LSD post hoc two-way ANOVA test). In accordance with this, the weight of iWAT, which was specifically measured, was also decreased in CR animals ($p<0.05$; LSD post hoc two-way ANOVA test), although the difference was more marked in male CR pups ($p<0.05$; LSD post hoc one-way ANOVA test). The decreased weight of iWAT compared to controls was associated with a parallel decrease in the diameter of adipocytes in both male and female animals ($p<0.05$; LSD post hoc two-way ANOVA test). In turn, leptin treatment throughout lactation had no demonstrable effects on any of the aforementioned parameters at this juvenile age. Regarding circulating parameters, no significant differences were found between groups concerning glucose and insulin levels, either due to calorie restriction during gestation or due to leptin treatment throughout lactation, as previously published (Konieczna et al. 2013). However, maternal calorie restriction during gestation led to a reduction of the plasma leptin levels in male rats, but not in females, which became partially reverted to the levels similar to controls due to leptin treatment throughout lactation ($p<0.05$; LSD post hoc one-way ANOVA test) (Konieczna et al. 2013). Analysis by considering males and females as a whole revealed a significant effect of maternal calorie restriction during gestation in decreasing T3 levels, but these became totally restored by leptin supplementation during the suckling period ($p<0.05$; LSD post hoc two-way ANOVA

Table 1. Weight-related and morphological traits and circulating parameters in the offspring of rats with free access to standard chow diet (control), the offspring of 20% calorie restricted dams during the first 12 days of pregnancy (CR), and CR rats daily supplemented with physiological doses of leptin throughout lactation (CR-Leptin).

	Males			Females			Males & Females
	Control	CR	CR-Leptin	Control	CR	CR-Leptin	ANOVA
Weight-related and morphological traits							
Body weight (g)	65.5 ± 1.1 **a**	59.6 ± 1.2 **b**	60.2 ± 1.2 **b**	61.7 ± 0.7 **a**	58.5 ± 1.2**b**	57.0 ± 1.0 **b**	S, G (a;b;b)
Body fat (%)	12.4 ± 0.3 **a**	10.1 ± 0.2 **b**	10.5 ± 0.2 **b**	12.2 ± 0.2 **a**	10.5 ± 0.2 **b**	10.5 ± 0.2 **b**	G (a;b;b)
iWAT							
Weight (mg)	559 ± 37 **a**	440 ± 21 **b**	431 ± 21 **b**	573 ± 38	521 ± 27	514 ± 28	S, G (a;b;b)
Adipocyte diameter (µm)	53.8 ±2.3 **a**	45.6 ± 1.4 **b**	45.0 ± 1.6 **b**	50.4 ±2.2 **a**	39.9 ± 1.7 **b**	40.7 ± 1.1 **b**	S, G (a;b;b)
Circulating parameters							
Glucose (mg/dL)	148 ± 4	145 ± 2	138 ± 4	149 ± 4	148 ± 5	142 ± 4	NS
Insulin (ng/L)	170 ± 24	204 ± 33	221 ± 59	248 ± 41	240 ± 49	163 ± 52	NS
Leptin (ng/L)	2206 ± 170 **a**	1798 ± 121 **b**	2042 ± 45 **a,b**	1689 ± 241	1461 ± 145	1414 ± 129	S
T3 (ng/ml)	4.31 ±0.35 **a**	3.12 ± 0.18 **b**	4.06 ±0.32 **a**	4.30 ± 0.37	3.73 ± 0.28	3.99 ± 0.15	G (a;b;a)

All parameters were determined on day 25. Data are mean ± S.E.M. For body weight, body fat content and weight of iWAT, n=16-17; for adipocyte diameter andcirculating parameters, n=6-8. Each group is made up of animals coming from at least six different litters. Statistical analyses were performed by considering males and females as a whole ($p \leq 0.05$; two-way ANOVA), and separately for each sex, to study individual differences between groups (controls, CR, CR-Leptin) ($p \leq 0.05$; one-way ANOVA). At any rate, data not sharing a common letter (a and b) are significantly different ($a \neq b$) ($p < 0.05$; LSD post hoc one-way and two-way ANOVA). Symbols: G, effect of group; S; effect of sex; NS, not statistically significant ($p < 0.05$; two-way ANOVA). Abbreviations:iWAT, inguinal white adipose tissue; T3,triiodothyronine.

test). This effect was more marked and significant by one-way ANOVA in male animals ($p<0.05$; LSD post hoc one-way ANOVA test).

TyrOH analysis in iWAT in offspring

TyrOH levels in iWAT were analyzed by Western blot and validated independently by immunohistochemistry (Figure 1).

Figure 1A shows specific protein levels of TyrOH in iWAT determined by Western blot. CR male animals showed a marked decrease of TyrOH levels in iWAT compared to their respective controls. Leptin supplementation throughout lactation totally normalized TyrOH levels to those of control males ($p<0.05$; LSD *post hoc* one-way ANOVA test). Unlike males, no differences were observed concerning TyrOH protein levels between female groups, either due to calorie restriction during gestation or due to leptin treatment throughout lactation. Based on these results, immunohistochemical analysis of TyrOH was performed only in iWAT of male animals (Figure 1B and 1C). In accordance with results obtained by western blot, CR male pups showed lower $TyrOH^+$ area in comparison to their controls, both expressed per tissue area (CR *vs* Control males; $p<0.05$; Student's *t*-test) and per adipocyte ($p<0.05$; LSD *post hoc* one-way ANOVA test). Notably, in CR-Leptin animals, the aforementioned decrease in $TyrOH^+$ area became normalized to the levels found in controls, both when referred to the tissue area (CR-Leptin *vs* Control males; $p<0.05$; Student's *t*-test) and to the number of adipocytes ($p<0.05$; LSD *post hoc* one-way ANOVA test).

mRNA expression levels of lipid metabolism-related genes (Atgl, Lipe, Cpt1b, Pgc1a, Lpl), and of Dio2 in iWAT in offspring

To ascertain whether the apparent changes in iWAT sympathetic innervation affected its function, the expression levels of lipid metabolism-related genes were determined in this tissue (Figure 2). Regarding expression levels of lipolytic genes, no differences in Atgl and Lipe mRNA levels were found between groups when analyzing male and female animals as a whole. However, single comparison between groups revealed that CR males exhibited decreased expression levels of both genes with respect to their controls (CR *vs* Control males; $p<0.05$; Student's *t*-test). Notably, in CR-Leptin group of males, mRNA levels of Atgl and Lipe became partially normalized (rise of 19.1% and 27.6%, respectively, compared to their CR counterparts).

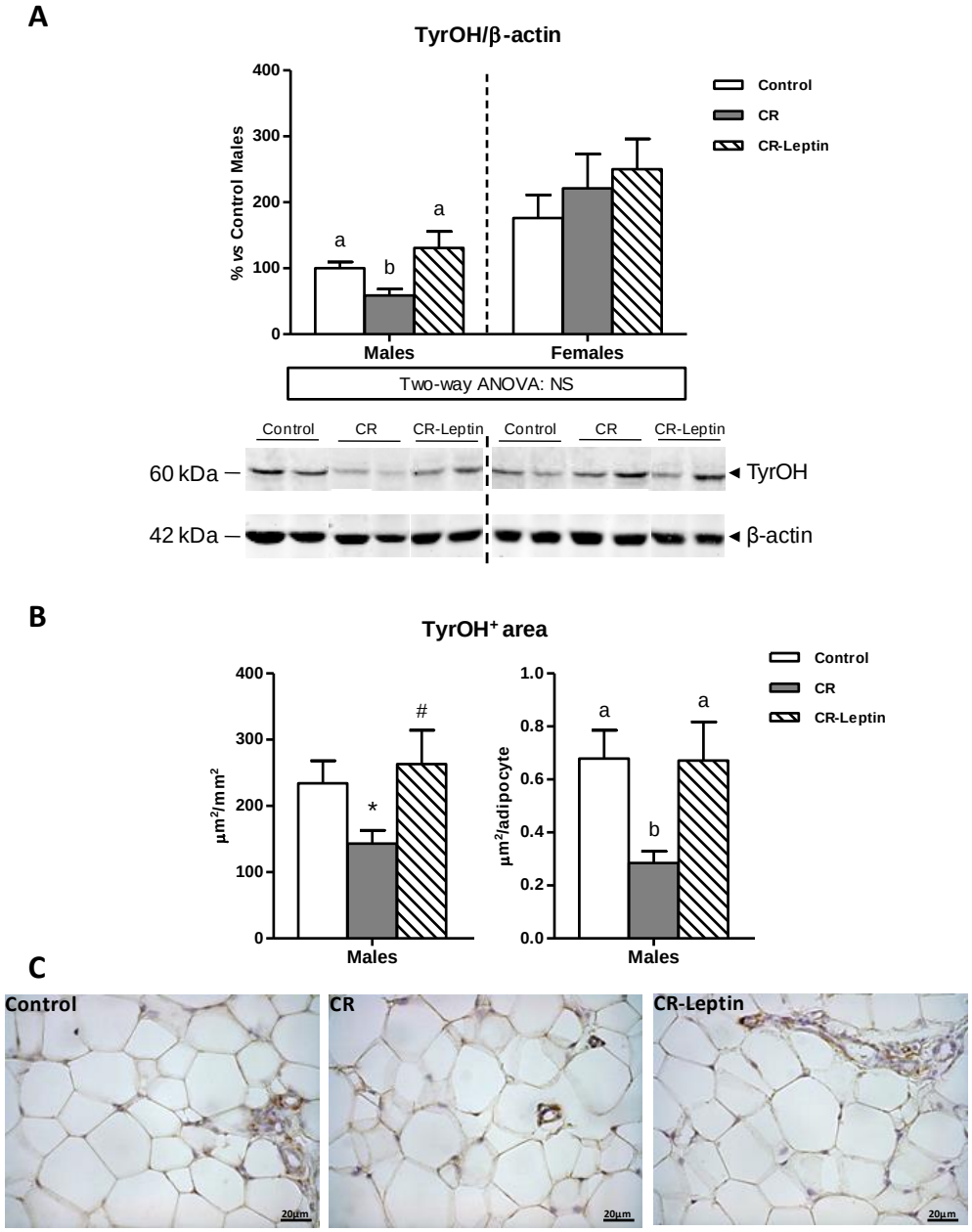

Figure 1. Specific abundance of TyrOH in iWAT of 25-day-old male and female offspring of dams with free access to standard chow diet (controls), the offspring of 20% calorie restricted dams during the first 12 days of pregnancy (CR), and CR rats daily supplemented with physiological doses of leptin throughout lactation (CR-Leptin). A. Protein levels of TyrOH were measured by Western blot, referred to those of ß-actin, and expressed as a percentage of the value of control male rats. Data are mean ± S.E.M. (n =8). Statistical analyses were performed by considering males and females as a whole ($p \leq 0.05$; two-way ANOVA), and separately for each sex, to study individual differences between groups (controls, CR, CR-Leptin) ($p \leq 0.05$; one-way ANOVA). At any rate, data not sharing a common letter (a and b) are significantly different (a≠b) ($p<0.05$; LSD post hoc one-way and two-way ANOVA). Symbols: NS, not statistically significant ($p<0.05$; two-way ANOVA). B. Immunoreactive TyrOH ($TyrOH^+$) area in male animals expressed as µm/mm^2 and as µm/adipocyte. Data are mean ± S.E.M. (n =5-7). Statistics: *, CR different from control group ($p<0.05$; Student's *t*-test); #, CR-Leptin different from CR group ($p<0.05$; Student's *t*-test); bars not sharing a common letter (a and b) are significantly different (a≠b) ($p<0.05$; LSD post hoc one-way ANOVA test). C. Representative iWAT sections of control, CR and CR-Leptin male animals immunostained for TyrOH. Scale bar: 20 µm. For each animal, 14–20 images of iWAT from light microscopy, coming from two nonconsecutive sections (7–10 fields per section), were chosen randomly and digitalized.

In addition, CR male animals showed a tendency to lower expression levels of the key gene involved in fatty acid oxidation, Cpt1b, in comparison to their controls (p=0.084; Student's *t*-test). Transcript levels of this gene were partially restored in CR-Leptin male animals (29.6% rise compared to CR males). A similar expression pattern was also found regarding the expression levels of Pgc1a, a key regulator of Cpt1b transcription. The decreased mRNA levels of Pgc1a in CR males (CR *vs* Control males; p<0.05; Student's *t*-test) tended to be restored due to leptin treatment throughout lactation (38.5% rise in its expression levels compared to CR males). Unlike males, female rats showed no significant differences regarding mRNA levels of Atgl, Lipe, Cpt1b and Pgc1a between groups. Considering males and females together, CR animals also showed lower Lpl mRNA levels than controls, which were partially recovered by the effect of leptin treatment during the suckling period (p<0.05; LSD *post hoc* two-way ANOVA test). When analyzing males and females separately, differences regarding expression levels of Lpl were more marked and significant only in males (p<0.05; LSD *post hoc* one-way ANOVA test).

In view of the differences between groups found in T3 plasma levels, mRNA expression levels of Dio2, a selenoenzyme that locally produces T3 via 5' deiodination of thyroxine (T4), were also measured in iWAT. Single comparison between groups, in males and females separately, revealed that CR females showed underexpression of Dio2 mRNA levels, in comparison to their controls (CR *vs* Control females; p<0.05; Student's *t*-test). Notably, mRNA levels of this gene became partially normalized by leptin treatment during the suckling period. In males, Dio2 mRNA levels followed the same pattern, although the differences did not reach statistical significance. Although analysis by considering males and females as a whole revealed that even the maternal calorie restriction during gestation did not significantly affect Dio2 mRNA levels, leptin-treated animals displayed overexpression of Dio2 mRNA levels in comparison to their CR counterparts (p<0.05; LSD post hoc two-way ANOVA test).

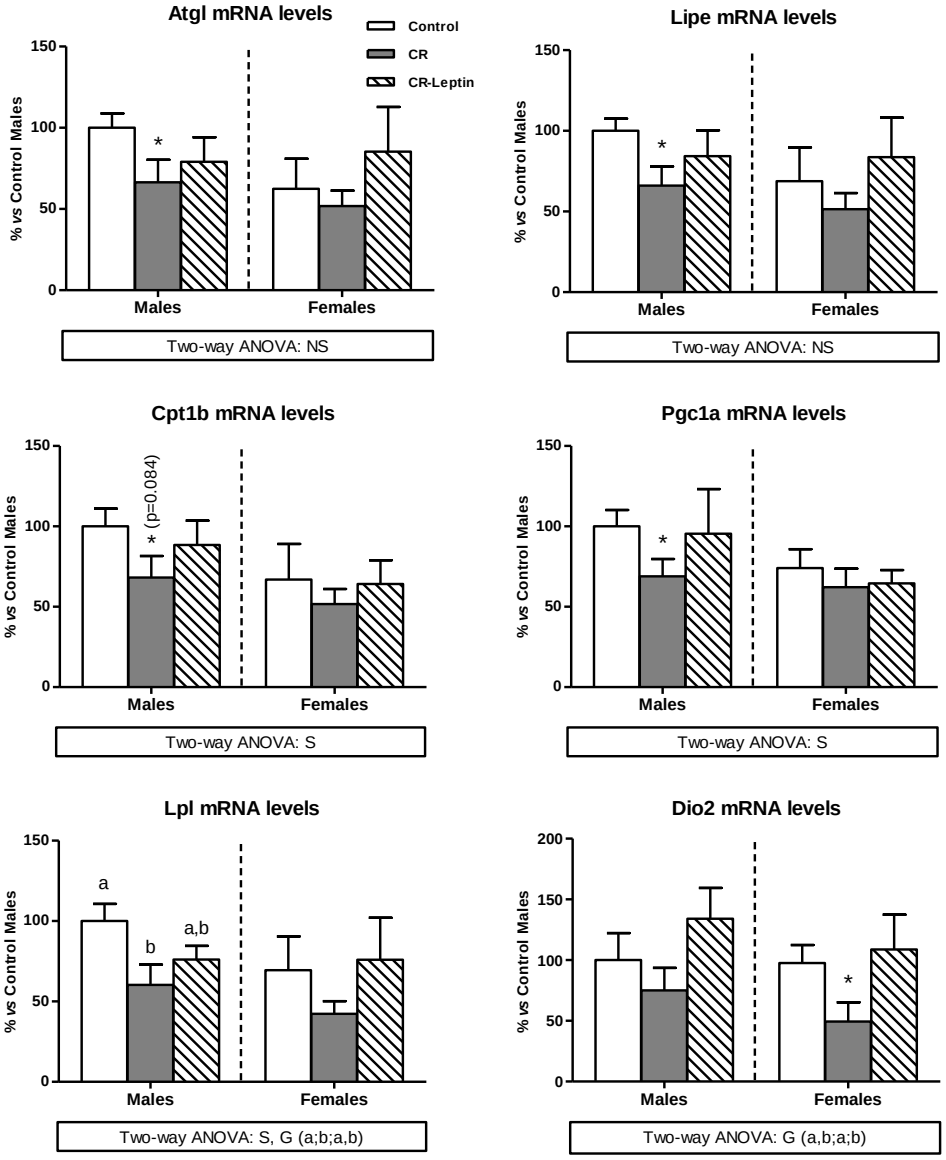

Figure 2. Expression levels of selected genes in iWAT in offspring. mRNA levels of Atgl, Lipe, Cpt1b, Pgc1a, Lpl and Dio2 in iWAT of 25-day-old male and female offspring of dams with free access to standard chow diet (controls), the offspring of 20% calorie restricted dams during the first 12 days of pregnancy (CR), and CR rats daily supplemented with physiological doses of leptin throughout lactation (CR-Leptin). mRNA levels were measured by RT-qPCR and expressed as a percentage of the value of control male rats. Data are mean ± S.E.M. (n=10-11, coming from at least six different litters). Statistical analyses were performed by considering males and females as a whole ($p \leq 0.05$; two-way ANOVA), and separately for each sex, to study individual differences between groups (controls, CR, CR-Leptin) ($p \leq 0.05$; one-way ANOVA). At any rate, data not sharing a common letter (a and b) are significantly different ($a \neq b$) ($p < 0.05$; LSD post hoc one-way and two-way ANOVA). Symbols: G, effect of group; S, effect of sex; NS, not statistically significant.

Discussion

We have previously shown that oral supplementation with physiological doses of leptin throughout lactation is able to revert, at least partly, some of the malprogrammed sequelae induced by poor prenatal nutrition, particularly by ameliorating central structures of the nervous system involved in the control of food intake and energy expenditure (Konieczna et al. 2013). However, without neglecting the remarkable role of the hypothalamus, WAT is also a key organ in the regulation of energy balance, hence, studies on WAT structure and function are of importance for identifying factors contributing to the greater propensity to obesity and hence potential therapeutic targets. Moreover, early programming of adipose tissue has been described to be determinant for the later functionality of the tissue (Garcia et al. 2011). Thus, here, we used a rat model of 20% mild maternal calorie restriction during the first part of gestation – which has been described to be enough to perturb sympathetic innervation of subcutaneous/inguinal WAT and hence to favour hyperplasia and fat accumulation in adulthood (Garcia et al. 2011) – to investigate whether leptin treatment may reverse malprogrammed effects on neuroanatomical structure and function of this fat depot.

Sympathetic innervation of iWAT was estimated by assessing TyrOH, the rate-limiting enzyme for catecholamines (epinephrine (EPI) and norepinephrine (NE)) synthesis (Giordano et al. 1996), using both biochemical (protein content) and morphological (tissue immunostaining) approaches. Both methods – determination of TyrOH content and tissue immunoreactivity for TyrOH – have been described to be a proper estimation of sympathetic innervation (Giordano et al. 2005) and have been previously experienced (Garcia et al. 2011). We have observed that moderate maternal calorie restriction during the first part of gestation altered development of SNS in iWAT of male animals, evidenced by decreased TyrOH protein levels and its immunoreactive area, with no demonstrable changes in females. These results are consistent with our previously reported results conducted in another cohort of animals (Garcia et al. 2011). Interestingly, here we show that oral supplementation with physiological doses of leptin throughout lactation was able to normalize TyrOH levels and its immunoreactive area in the offspring of calorie restricted male animals.

It must be highlighted that the effects of leptin treatment increasing WAT sympathetic innervation (and hence recovering the normal pattern) were dependent upon prior

developmental programming. Here, it is confirmed that female animals seem to develop a certain resistance to the detrimental consequences of calorie restriction during gestation concerning iWAT sympathetic innervation, as previously described (Garcia et al. 2011), which may also be related to the lower detrimental effects of this condition in adulthood in comparison to males (Palou et al. 2010). Thus, it is not surprising that leptin treatment did not affect neuroanatomical structure of iWAT in female pups.

The SNS influences the growth or maintenance of adipose tissue mass. iWAT sympathetic denervation has been described to decrease NE content and increase fat pad mass and fat cell number (Youngstrom and Bartness 1998). In a previous study conducted in another cohort of animals (Garcia et al. 2011), but applying the same model of moderate gestational calorie restriction, the decrease in sympathetic innervation in iWAT occurring in male animals was associated to the development of hyperplasia and increased fat mass. This altered phenotype was found in adulthood and under high-fat diet conditions. As the present study was conducted until the age of 25 days, the reduction of iWAT mass (only in males) and adipocyte size (diameter) found at this early age might be a consequence of prenatal food deprivation, as these animals were just allowed to eat independently. Oral supplementation with physiological doses of leptin throughout lactation did not show any apparent effect on the growth of this depot at this juvenile age. However, the restoration of iWAT sympathetic innervation seen in leptin-treated male animals would be expected to protect against the early programmed abnormalities previously described (Garcia et al. 2011) in the development of this fat depot.

Moreover, it is well established that sympathetic innervation in WAT stimulates lipid mobilization (Bartness and Bamshad 1998) by activation of key enzymes in the control of lipolysis in WAT, such as ATGL and HSL. Increased release of NE from sympathetic nerves innervating adipose tissue activates β_3-adrenoreceptors, which interacts with G-protein to stimulate adenylate cyclase. Increase in intracellular levels of cAMP activates PKA, which phosphorylates and activates HSL (Holm 2003). In turn, ATGL – which works in conjunction with HSL, is the initiator lipase for NE-stimulated lipolysis and may be rate limiting in the mobilization of cellular fat depots. ATGL activity, unlike that of HSL – is not dependent on PKA phosphorylation (Bartness et al. 2010). Alterations in adipose tissue innervation were expected to trigger changes in the capacity to mobilize fat stores. Here, we have shown that partial sympathetic

dennervation of iWAT occurring in CR male animals was accompanied by a decrease in mRNA expression levels of Atgl and Lipe genes, which encode for ATGL and HSL proteins, respectively. Unlike males, changes in mRNA levels of these aforementioned genes were not apparent in female CR animals. A similar expression pattern was found in male pups for genes related to β-oxidation. Although CR male animals exhibited only a trend to lower mRNA levels of Cpt1b – which catalyzes the rate limiting step in mitochondrial fatty acids oxidation (Kerner and Hoppel 2000) – mRNA levels of Pgc1a, a crucial transcriptional regulator of genes involved in fatty acid oxidation (Kok et al. 2013), were significantly diminished in comparison to their controls. It has been described that activation of β_3-adrenoreceptors induces upregulation of genes involved in fatty acid oxidation and mitochondrial electron transport activity (Granneman et al. 2005; Bogacka et al. 2007). Therefore, the altered sympathetic innervation observed in iWAT of CR male pups may account for lessened oxidative capacity of their adipocytes. Notably, oral supplementation with physiological doses of leptin throughout lactation was also able to correct, at least partly, expression levels of genes involved in lipolysis (Atgl, Lipe) and β-oxidation (Cpt1b, Pgc1a) in WAT, as their mRNA levels rose to levels not different to those of control males. Restoration of phenotypic expression in adipocytes involved in catabolic activity of WAT may result in better handling and partitioning of excess fuel, and thus prevent the programmed predisposition to fat accumulation and other metabolic abnormalities that CR animals, particularly males, undergo in adult life (Palou et al. 2010; Palou et al. 2012).

Impaired catabolic activity of WAT, as a result of maternal calorie restriction during lactation, was accompanied by lower mRNA expression levels of Lpl. Notably, these alterations were found in both male and female CR animals, although they were more marked in male pups. LPL supplies WAT with fatty acids from circulating triacylglycerol-rich lipoproteins, which are stored as triacylglycerol (Braun and Severson 1992). In this sense, we have previously described that both male and female CR animals exhibited elevated (fed and fasted) circulating triacylglycerols levels than their controls, both at juvenile age and in adulthood (Palou et al. 2010). Furthermore, in WAT, LPL activity has been shown to be increased after cAMP activation (Ballart et al. 2003). As the SNS controls lipid metabolism through the intracellular messenger cAMP, its impairment is expected to account for downregulation of Lpl mRNA levels in CR male animals. However, unlike males, the effects in CR females cannot be directly

explained by lower sympathetic innervation. In turn, oral supplementation with physiological doses of leptin throughout lactation to the offspring of undernourished dams partially restored Lpl mRNA expression to the levels of their controls, in both males and females. In rats born from adequately nourished dams during gestation, oral supplementation with the same doses of leptin during lactation, as used here, was previously shown to improve the lasting effects of HF-diet feeding on adipose tissue by increasing mRNA expression levels of genes involved in energy uptake and oxidation (Priego et al. 2010).

In addition to the SNS, the adipose tissue is also a target of thyroid hormones. These hormones regulate adipogenesis and related processes such as lipogenesis and lipolysis, however the mechanisms involved in these actions in WAT have been less explored to date. Thyroid hormones also increase LPL activity (Pucci et al. 2000). Therefore, the SNS and thyroid hormones represent two pathways, which act simultaneously for the induction of catecholamine signalling cascade and lipolysis activation in WAT. Metabolic effects of thyroid hormones in tissues depend on intracellular levels of the biologically active form of T3, which is produced by deiodination of thyroxine (T4). This reaction is catalyzed by iodothyronine 5'-deiodinases, DIO1 and DIO2, and reversed by DIO3 (Dentice and Salvatore 2011). DIO1 is highly present in liver, and its activity is increased by circulating T3 (Bianco et al. 2002), whereas DIO2 activity is predominantly present in the pituitary, brain, and BAT, and plays a crucial role in the local generation of T3 in the tissues (Bianco et al. 2002). Expression and activity of DIO1 and DIO2 has also been evidenced in WAT (Lado-Abeal et al. 2010; Calvo and Obregon 2011), and DIO2 activity has been proposed to play a crucial role in the development of the tissue, as well as in thermogenesis and lipogenesis (Calvo and Obregon 2011). In the present study, we found that CR animals, particularly males, displayed lower T3 plasma levels compared to controls, in accordance with previously published results in animals exposed to the same conditions during gestation (Palou et al. 2014). Severe (50%) maternal calorie restriction during pregnancy has also been described to lead to diminishment of plasma T3 levels in male offspring (Anguita et al. 1993). Here, the aforementioned decrease in T3 levels occurring in CR animals was accompanied by lower mRNA expression levels of Dio2 in WAT, particularly in females, suggesting lower T3 availability in this tissue. Therefore, since WAT sympathetic innervation in CR female animals was not apparently affected by maternal

calorie restriction during gestation, the downregulation of Lpl found in iWAT of male and female animals compared to controls could be tentatively attributed to altered thyroid hormone signalling. Notably, in CR-Leptin animals, the decreased plasma levels of T3 became restored, which was accompanied by a rise in Dio2 mRNA levels, when comparing to CR animals. This may account for the normalization of the impaired capacity to mobilize fat storage in CR animals, particularly males, and would be expected to prevent the programmed predisposition to fat accumulation which these undergo in adult life (Palou et al. 2010; Palou et al. 2012). This may also account for the normalisation of Lpl mRNA levels in both males and females.

All in all, these results point out that sex-specific early-programmed effects of calorie restriction during gestation on WAT innervation and function are reversible by oral leptin treatment throughout lactation. This study brings evidence supporting the relevance of the intake of leptin during lactation, a specific compound of breast milk with beneficial health effects, which might be worth considering when searching for strategies to treat and/or prevent the programmed trend to obesity caused by inadequate fetal nutrition.

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MANUSCRIPT 5

Leptin supplementation in suckling rats reverts blood cell transcriptomic-based potential early biomarkers of adverse programming effects associated to gestational calorie restriction

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Title page

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Conflict of interest: AP, CP and JS are authors of a patent held by the University of the Balearic Islands on the use of leptin as an essential nutrient during lactation. The name of the patent is "Use of leptin for the prevention of excess body weight and composition containing leptin" (WO 2006089987 A1) (priority data: 23 Feb 2005).

Abstract

Modest maternal undernutrition during gestation in rats may program the offspring to develop later pathologies. Oral administration of physiological doses of leptin throughout lactation could revert some of these alterations. Using these animal models, we aimed to identify early transcriptome-based biomarkers, indicators of both programmed susceptibility to later disorders and response to neonatal leptin treatment in peripheral blood mononuclear cells (PBMCs), an easily accessible surrogate tissue. Male and female rats belonging to 3 groups were studied: the offspring of *ad libitum* fed dams (controls), the offspring of 20% calorie restricted dams during the first part of pregnancy (CR), and CR rats supplemented with physiological doses of leptin throughout lactation (CR-Leptin). Animals were sacrificed on postnatal day 25 and PBMCs were isolated. Whole genome microarray analysis was performed in PBMCs from male rats. 224 known genes were differentially expressed between control and CR animals ($p \leq 0.010$). Leptin treatment in CR males normalised the expression of 218 of these genes to control levels. Among them, 22 were selected for confirmation in q-PCR as the most fully reversed ($p \leq 0.010$). Of these 22 genes, q-PCR analyses in PBMCs from males and females revealed *Crmp1*, *Gla*, *Gls*, *Lrp11*, *Paox*, *Tmsb4x* and *Ubash3b* as particularly interesting, because increased expression levels found in CR pups were totally reverted by leptin treatment in both genders. In conclusion, leptin supplementation throughout lactation is able to revert most of the transcript-based potential early biomarkers identified in PBMCs associated to poor nutrition during pregnancy. These markers may be useful for early identification and subsequent monitoring of individuals who are at risk of later diseases and would specifically benefit from the intake of appropriate amounts of leptin during lactation.

Keywords: leptin treatment; gestational calorie restriction; obesity; early biomarkers; PBMCs; developmental programming

Introduction

There is an accumulating body of evidence showing that maternal nutrition during the perinatal period has significant effects on fetal growth and can exert powerful influences upon long-term health and well-being (Sullivan and Grove 2010; Pico et al. 2012). In this view, emerging evidence in human and animal models associates maternal calorie-restriction during pregnancy with adverse health outcomes in adult offspring, mainly related, but not restricted, to the development of obesity, cardiovascular diseases and type II diabetes, particularly when exposed postnatally to obesogenic conditions (Palou et al. 2012; Palou et al. 2010; Ravelli et al. 1976; Vickers et al. 2000). Although the factors involved in the programming effects are not clearly known, the role of the hormone leptin is receiving special attention (Pico et al. 2007; Jones 2010).

Leptin is mainly produced by the adipose tissue, but is also naturally present in significant amounts in breast milk (Houseknecht et al. 1997). In addition to the well established role of leptin in the regulation of energy homeostasis in adulthood (Schwartz et al. 2000; Arora and Anubhuti 2006), leptin has been shown to play a crucial neurotrophic role in neonates by programming hypothalamic circuits formation during a neonatal restricted window, which coincides with an increase in plasma leptin levels, the so called leptin surge (Bouret et al. 2004; Bouret and Simerly 2006). In this sense, our group and others have evidenced that maternal undernourishment during gestation in rodents, which is associated to disturbed neonatal leptin surge (Yura et al. 2005; Palou et al. 2012), perturbs hypothalamic structure and function (Delahaye et al. 2008; Ikenasio-Thorpe et al. 2007; Garcia et al. 2010), and hence results in a greater propensity for obesity and insulin resistance development in adulthood. We have described for the first time that leptin intake during lactation, orally administered at physiological doses comparable to those present in breast milk, protects againsts obesity and related alterations later on in adult life (Pico et al. 2007; Sanchez et al. 2008). More recently, we have also shown that daily administration of oral leptin at physiologic doses throughout lactation to the offspring of moderate calorie restricted dams during gestation, reverted, at least partly, most of the developmental effects on hypothalamic structure and function, hence making this metabolic malprogramming reversible to some extent (Konieczna et al. 2013). Vickers et al. (Vickers et al. 2005; Vickers et al. 2008) also evidenced that a daily subcutaneous injection of leptin into neonatal rats born

to undernourished mothers prevented the development of a programmed trend towards obesity and other metabolic alterations in later life. Positive effects of oral leptin during lactation are not restricted to the offspring of calorie-restricted dams. We have described that leptin supplementation to the offspring of adequately nourished dams prevents age-related metabolic alterations associated to the intake of a high-fat diet (Pico et al. 2007; Sanchez et al. 2008; Priego et al. 2010). Other studies – which addressed the effects of early postnatal diet on healthy, lasting outcomes – evidenced that infants who are breastfed are less likely to become obese than those who are exclusively formula fed (von Kries et al. 1999; Armstrong and Reilly 2002), and a negative association has been found between maternal milk leptin levels and body weight increase of infants (Miralles et al. 2006), which has been confirmed by other groups (Schuster et al. 2011; Doneray et al. 2009). Thus, leptin may be considered as an essential factor during lactation in the protection against later overweight, obesity and its related metabolic disorders in later life, and may also be worth considering when searching for strategies to reverse programmed susceptibility to obesity acquired by fetal undernutrition (Pico et al. 2011; Vickers and Sloboda 2012).

Knowing the risk of obesity and related metabolic alterations appears to be essential to implement effective prevention strategies. In this regard, identification of early biomarkers may provide a diagnostic tool to detect and monitor emerging perturbations related to obesity. Biomarkers conventionally used to predict the risk of obesity and its co-morbidities development mainly involve body weight, BMI, blood pressure, lipid profile, plasma glucose and insulin levels. Bearing in mind the complexity of the mechanisms contributing to obesity and its related pathologies, there is a growing need for multiple novel biomarkers beyond the prediction of traditional metabolic risk factors (Musaad and Haynes 2007; Schneider et al. 2012; Balagopal et al. 2011). Application of early transcript-based metabolic markers might be more beneficial, providing more information regarding disease or physiological changes occurring in the body, as well as giving more time to intervene in the prevention of a future disease (de Mello et al. 2012; Konieczna et al. 2014). Studies on molecular mechanisms revealing effects of diet on health usually imply invasive tissue biopsies (Viguerie et al. 2005). Peripheral blood mononuclear cells (PBMCs) provide an attractive alternative that can be assessed in humans, because they can be easily and repeatedly collected in sufficient quantities (de Mello et al. 2008; Konieczna et al. 2014; Oliver et al. 2013; Caimari et al. 2010). These

cells are able to respond to internal and external signals, and thereby have been proposed as a source of biomarkers of health and disease, as their gene expression profile may reflect the physiological and pathological state of the organism (Bouwens et al. 2007; Manoel-Caetano et al. 2012; Burczynski and Dorner 2006; Oliver et al. 2013; Caimari et al. 2010).

Therefore, in the present study, we used whole genome transcriptome profiling of PBMCs of pups from calorie-restricted dams during gestation to identify early biomarkers of programmed susceptibility to obesity-related chronic diseases and to determine whether oral leptin treatment with physiological doses throughout lactation is able to revert these markers of developmental malprogramming.

Materials and methods

Animals and experimental design

The animal protocol followed in this study was reviewed and approved by the Bioethical Committee of the University of the Balearic Islands and guidelines for the use and care of laboratory animals of the University were followed.

The study was conducted on male and female Wistar rat pups from 17 different litters following the protocol during pregnancy and lactation described below. Animals were housed under standard conditions, that is, controlled temperature (22 °C), the normal 12-h light and 12-h dark cycle, free access to tap water and a standard laboratory rodent chow diet (3.3 kcal/g, with 8% calories from fat; Panlab, Barcelona, Spain), unless specified. Virgin female Wistar rats (body weight 217 g - 244 g) were mated with male rats (Charles River Laboratories, Barcelona, Spain). Day of conception (day 0 of pregnancy) was determined by examination of vaginal smears for the presence of sperm. Pregnant rats were divided into two groups: control dams (n=7 animals) fed *ad libitum* with standard chow diet, and calorie restricted dams (CR-dams) (n=10 animals) fed with 20% calorie restriction from day 1 to day 12 of gestation, as previously described (Palou et al. 2010). After the calorie restriction period, all dams were fed *ad libitum*, and food intake was measured.

On day 1 after delivery, excess pups in each litter were removed to keep 10 pups per dam (five males and five females, when possible). Pups of both sexes born from CR-dams were randomly assigned into two groups: CR and CR-Leptin. CR-Leptin animals

were supplemented, each day throughout lactation with an oral solution of recombinant murine leptin (PeproTech, London, UK) dissolved in water by using a pipette. The amount of leptin given to animals was progressively increased from 1 ng of leptin on day 1, to 43.8 ng of leptin on day 20 of life, as previously described (Pico et al. 2007). CR pups and the offspring of control dams (controls) received the same volume of the vehicle (water).

Pups were weaned at 21 days of life, and 35 pups from control group (18 males and 17 females), 34 from CR group (17 males and 17 females), and 33 from CR-Leptin group (17 males and 16 females) were housed in groups of two animals, and fed on a standard chow diet.

On day 25, pups were sacrificed under *ad libitum* feeding conditions by decapitation, during the first 2 h of the beginning of the light cycle. Samples of trunk blood were collected for PBMCs isolation and gene expression analysis (n = 10-11 animals/group) and for plasma circulating parameters determination (n = 6-8, per group). Blood samples were collected in heparinized containers, then centrifuged at 1000*g* for 10 min to obtain the plasma, and stored at −20 ºC until analysis. Collection of blood and subsequent PBMCs isolation is described in the section below. Animals used for the different analyses were from at least six different litters.

PBMC isolation

Trunk blood samples of control, CR and CR-Leptin rats collected at the age of 25 days under *ad libitum* feeding conditions, were used to isolate PBMCs. Peripheral blood samples were collected using EDTA (final concentration of 3-4 mM) as anticoagulant, and then diluted with an equal volume of buffered saline (isosmotic). PBMCs were immediately isolated by OptiPrep density-gradient separation (Sigma Aldrich Química SA, Madrid, Spain) according to the manufacturer's instructions.

Total RNA isolation

Total RNA was extracted from PBMCs of control,CR and CR-Leptin male and female animals by EZNA® TOTAL RNA kit I (Omega Bio-Tek Inc., Norcross, GA, USA) following the manufacturer's instructions.

Microarray processing

For microarray analysis, RNA from PBMC samples obtained from male offspring of controls, CR and CR-Leptin animals at the age of 25 days were used (n=10-11/group). RNA samples were analyzed on Agilent 2100 Bioanalyzer with RNA 6000 Nano chips (Agilent Technologies, Barcelona, Spain). To ensure the high quality of RNA, only samples having a RIN number ≥ 8 were used for microarrays (n=8/group). Then, 80 ng of RNA from each sample was reverse transcribed to complementary DNA (cDNA) using the Agilent Low Input Quick Amp Labeling kit (Agilent Technologies), according to the manufacturer's protocol. Half of the cDNA sample (10 µl) was used for the linear amplification of RNA and labeling with cyanine-3 (Cy3) or Cy5. For these reactions, half of the amounts indicated by the manufacturer were used (van Schothorst et al. 2007). Transcription and labeling were carried out at 40 °C for 2 h. Then, the labeled and amplified cRNA samples were purified using Qiagen Rneasy MiniSpin columns (Qiagen, Madrid, Spain). The incorporation of dyes and cRNA concentration was measured using the NanoDrop ND 1000 spectrophotometer (NanoDrop Techonologies, Ins., Wilmington, DE). Of the 24 samples, 20 were used for microarray analysis. Four samples (2 samples in the CR and 2 samples in the CR-Leptin group) were excluded because of a low yield (<825 ng) and low specific activity (<8.0 pmol Cy3 or Cy5 per mg of cRNA). Subsequently, each sample containing 825 ng of cRNA labeled with Cy5 and 825 ng of Cy3 pool were hybridized on 4x44K G2519F rat whole genome Agilent microarrays (Agilent Technologies) for 17 h at 65 °C in hybridization chambers in an oven rotating at 10 rpm (Agilent Technologies). After hybridization, the arrays were washed using Gene Expression (GE) Wash Buffer Kit (Agilent Technologies). The arrays were rinsed in GE Wash Buffer 1 for 1 min, GE Wash Buffer 2 for 1 min, followed by acetonitrile for 10 s, and finally with a Stabilization and Drying Solution for 30 s at room temperature, according to the manufacturer's protocol (Agilent Technologies).

Microarray data analysis

The arrays were scanned with an Agilent Microarray Scanner (Agilent Technologies). Scanned images were examined for visible defects and proper grid alignment. The intensities of the signals from each spot were quantified, and the raw data were extracted using Feature Extraction Software version 10.10.1.1 (Agilent Technologies).

Background correction and normalization were performed within the Babelomics platform (http://www.babelomics.org), a suite of web tools for microarray data analysis. Differential gene expression between groups of animals (CR *vs* controls, CR-Leptin *vs* CR, and CR-Leptin *vs* controls) was assessed using the limma package from Bioconductor, implemented into Babelomics web platform (Medina et al. 2010). Fold changes (FC) were also calculated in this platform. The threshold of significance for this statistical test was set at $p \leq 0.010$. Subsequently, a statistically generated list of genes was manually analyzed in regard to their biological information, obtained with the use of available databases (Genecards, KEGG, NCBI, Reactome, UniProt, USCN, WikiPathways) based on key biological domains, such as molecular function and biological process.

Real-time quantitative RT-polymerase chain reaction (RT-qPCR) analysis

To validate microarray data, mRNA expression levels of selected genes: collapsin response mediator protein 1 *(Crmp1)*; digestive organ expansion factor homolog (zebrafish) *(Diexf)*; fos-like antigen 1 *(Fosl1)*; galactosidase, alpha *(Gla)*; glutaminase *(Gls)*; low density lipoprotein receptor-related protein 11 *(Lrp11)*; polyamine oxidase (exo-N4-amino) *(Paox)*; ring finger protein 10 *(Rnf10)*; selenium binding protein 1 *(Selenbp1)*; solute carrier family 7 (amino acid transporter light chain, L system), member 5 *(Slc7a5)*; thymosin beta 4, X-linked *(Tmsb4x)*; ubiquitin associated and SH3 domain containing, B *(Ubash3b)* were measured by RT-qPCR in PBMC RNA samples of control, CR and CR-Leptin male animals. In addition, mRNA expression levels of the same set of genes were analyzed in PBMC samples of control, CR and CR-Leptin female animals to verify the manner of their response to the treatments used.

For RT-qPCR analysis, 0.05 µg of PBMC total RNA was used for reverse transcription by using iScript™ cDNA synthesis kit (Bio-Rad Laboratories, S.A., Madrid, Spain) according to the manufacturer's protocol.

Real-time PCR was performed using the Applied Biosystems StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Madrid Spain) with Power SYBER Green PCR Master Mix (Applied Biosystems). Each PCR was performed from 1/5 dilution of the cDNA product and forward and reverse primers (5 µM each). Primer sequences and products for the different genes are described in Table 1. All primers were purchased from Sigma Genosys (Sigma Aldrich Química SA, Madrid, Spain). After an initial Taq

activation at 95 °C for 10 min, PCR was performed using 40 two-temperature cycles with the following cycling conditions: 95 °C for 15s and 60 °C for 1 min. To verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The values for the threshold (Ct) were calculated by the instrument's software (StepOne Software v2.2.2), and the relative expression of each mRNA was calculated as a percentage of male control rats, using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001) with proteasome (prosome, macropain) subunit, alpha type 6 *(Psm6)* as reference gene. It was chosen because our microarray data showed equal expression of this gene across all groups.

Table 1. Nucleotide sequences of primers and amplicon size used for qRT-PCR.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon (bp)
Crmp1	*TGATTGTTCCTGGTGGAGTG*	*GGATTTGGTGTCTGCTGCTT*	248
Diexf	*TTCTACGACAGGGTTTCCAAG*	*GCCATCTTCACCATTCATTTC*	258
Fosl1	*GCAGAAACCGAAGAAAGGAA*	*CTGGAGAAAGGGAGATACAAGG*	261
Gla	*CCCGAGAGGGATTCAAAG*	*TACCCCAGTCAGCAAATGTC*	199
Gls	*GGAGGGAAGGTTGCTGATTA*	*AGGACTGAAGACAAAAGGGAAC*	133
Lrp11	*ACAGACGACCACGCCATT*	*CCTGGGAAGCACAGTCACA*	198
Paox	*TGGCTGTCCTGAATACCTTCTT*	*TCAAAAACCATCACCTCCTTG*	190
Psm6	*TGGCTATGAGATTCCTGTGG*	*CTGTCTGCTTCACTCCTGCT*	206
Rnf10	*GGGGGAAAAGAAACAAGTGG*	*AAGGTGTCAGGGTCAGCAAA*	124
Selenbp1	*TGACCGCTTCCTCTACTTCA*	*CTCGTTTTCCCTTGACCACT*	194
Slc7a5	*CCTGCCTCTGCGTGCTACT*	*CCCTTGTCCTATGTCCTTTCC*	155
Tmsb4x	*GCTCCTTCCAGCAACCAT*	*GGGGCAGCACAGTCATTT*	278
Ubash3b	*ACTTCATCGGGCTCTTTGTG*	*TGTTCTGGGCGAGTTTCTCT*	189

Abbreviations: Collapsin response mediator protein 1 *(Crmp1)*; Digestive organ expansion factor homolog (zebrafish) *(Diexf)*; Fos-like antigen 1 *(Fosl1)*; Galactosidase, alpha *(Gla)*; Glutaminase *(Gls)*; Low density lipoprotein receptor-related protein 11 *(Lrp11)*; Polyamine oxidase (exo-N4-amino) *(Paox)*; Proteasome (prosome, macropain) subunit, alpha type 6 *(Psm6)*; Ring finger protein 10 *(Rnf10)*; Selenium binding protein 1 *(Selenbp1)*; Solute carrier family 7 (amino acid transporter light chain, L system), member 5 *(Slc7a5)*; Thymosin beta 4, X-linked *(Tmsb4x)*; Ubiquitin associated and SH3 domain containing, B *(Ubash3b)*.

Statistical analysis

Data are reported as mean ± standard error (SEM). The statistical analysis of microarray data has been described in detail in the section referred to microarray data analysis. Multiple comparisons were assessed by one-way (separately for each sex) and two-way ANOVA, to study individual differences between groups (controls, CR, CR-Leptin), followed by least significance difference (LSD) *post hoc* test. The data were confirmed

for equality of variances by Levene's test ($p< 0.05$). Single comparisons between groups were assessed by Student's *t* test. $P<0.05$ was the threshold of significance, unless stated. All the analyses were performed with SPSS Statistics 19.0 (SPSS, Chicago, IL).

Results

Anthropometric measurements and circulating parameters in the offspring

Description of the phenotype of pups at birth and after weaning has been previously published (Konieczna et al. 2013). Here we summarize data related to body weight, body fat content and blood parameters (Table 2). Maternal calorie restriction during gestation did not affect body weight at birth. However, during the suckling period, CR pups gained less weight and exhibited lower body weight and fat content than their controls after weaning ($p<0.05$; LSD *post hoc* one-way ANOVA test). Leptin treatment throughout lactation had no demonstrable effects on the mentioned parameters ($p<0.05$; LSD *post hoc* one-way ANOVA test).

Analyses of circulating levels of glucose and insulin at postnatal day 25 showed no statistical differences between groups either due to calorie restriction during gestation or due to leptin treatment throughout lactation. However, in males, but not in females, maternal calorie restriction during gestation led to a significant reduction of plasma leptin levels, which became partially reverted to the levels similar to controls due to leptin treatment throughout lactation ($p<0.05$; LSD *post hoc* one-way ANOVA test).

Gene expression in PBMCs of male animals at the age of 25 days based on whole-genome microarray analysis

Whole genome microarray analysis was performed in PBMCs of 25 day-old male animals belonging to the 3 groups of animals (control, CR, and CR-Leptin). Of the 45,220 probes tested, 36,122 probes remained after background correction, normalization and merged replicated clones and were further taken into account. In total, 473 unique genes were found to be significantly different between control and CR male animals ($p\leq0.010$; limma *t*-test). Using available databases (Genecards, KEGG, NCBI, Reactome, UniProt, USCN, WikiPathways), these genes were classified into several biological processes according to their function. 249 genes were unknown and were not considered.

Table 2. Offspring parameters: anthropometric measurements and circulating parameters in the offspring of rats with free access to standard chow diet (control), the offspring of 20% calorie restricted dams during the first 12 days of pregnancy (CR), and CR rats daily supplemented with physiological doses of leptin throughout lactation (CR-Leptin).

	Males			Females		
	Control	**CR**	**CR-Leptin**	**Control**	**CR**	**CR-Leptin**
Anthropometric measurements						
Body weight, day 1 (g)	6.57 ± 0.18	6.37 ± 0.11	6.45 ± 0.12	6.38 ± 0.08	6.15 ± 0.13	6.18 ± 0.12
Body weight, day 21 (g)	47.0 ± 0.9 **a**	43.2 ± 0.9 **b**	43.9 ± 0.9 **b**	45.4 ± 0.6 **a**	42.2 ± 0.8 **b**	42.8 ± 0.8 **b**
Body weight, day 25 (g)	65.5 ± 1.1 **a**	59.6 ± 1.2 **b**	60.2 ± 1.2 **b**	61.7 ± 0.7 **a**	58.5 ± 1.2 **b**	57.0 ± 1.0 **b**
Body fat, day 25 (%)	12.4 ± 0.3 **a**	10.1 ± 0.2 **b**	10.5 ± 0.2 **b**	12.2 ± 0.2 **a**	10.5 ± 0.2 **b**	10.5 ± 0.2 **b**
Circulating parameters (day 25)						
Glucose (mg/dL)	148 ± 4	145 ± 2	138 ± 4	149 ± 4	148 ± 5	142 ± 4
Insulin (ng/L)	170 ± 24	204 ± 33	221 ± 59	248 ± 41	240 ± 49	163 ± 52
Leptin (ng/L)	2206 ± 170 **a**	1798 ± 121 **b**	2042 ± 45 **a,b**	1689 ± 241	1461 ± 145	1414 ± 129

Body weight was measured on postnatal days 1, 21 and 25. The other parameters were determined on day 25. Data are mean ± S.E.M. For body weight at different days and body fat content, n = 16-17; for circulating parameters, n = 6-8. Each group is made up of animals coming from at least six different litters. Statistics: data not sharing a common letter are significantly different (a≠b) ($p < 0.05$; LSD *post hoc* one-way ANOVA test).

From the remaining 224 known genes, 58 exhibited down-regulation and 166 up-regulation (Figure 1). The processes with the highest number of genes differentially expressed were related to transcription/translation machinery, immune system, signaling, cell turnover and metabolism of proteins and polyamines (33, 25, 23, 20 and 20 genes, respectively). Other processes with a notable number of genes whose expression was altered were related to transport, metabolism of lipids, cytoskeleton, and sensory perception, nervous system and neural signaling (15, 13, 10, 10, 9 and 8 genes, respectively). The remaining genes were related to cell communication, blood, metabolism of carbohydrates, epigenetic modification, metabolism of nucleotides, central and redox metabolism (7 or less genes involved in each of the processes). Other biological processes such as fertilization, detoxification, angiogenesis, and embryonic development, were grouped with the name 'others'.

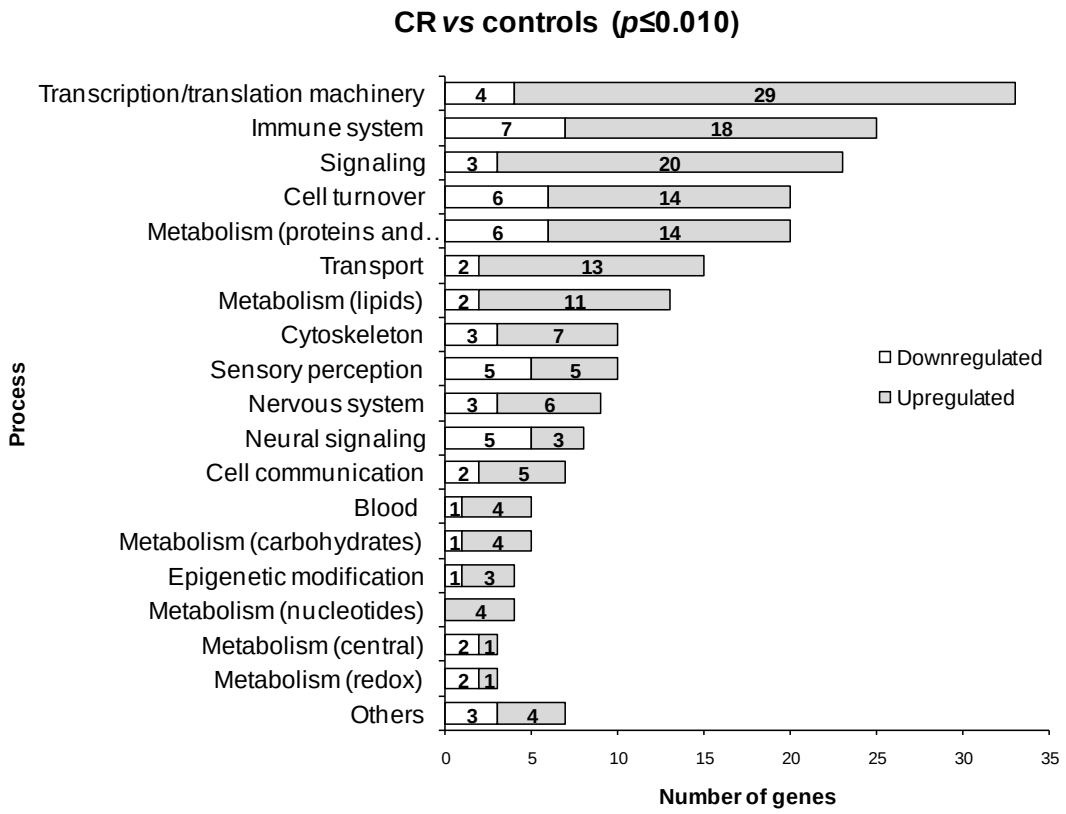

Figure 1. Microarray data classification into biological processes of the genes differentially expressed in PBMCs between male offspring of control and calorie-restricted dams during gestation (CR) at the age of 25 days. Threshold of significance was set at $p \leq 0.010$ (limma *t*-test). The number of genes down- or up-regulated is indicated for each group of genes.

Subsequently, we performed statistical analyses to compare mRNA expression levels between CR and CR-Leptin groups and between CR-Leptin and control groups. Of the 224 known genes differently expressed in CR pups *vs* controls ($p \leq 0.010$; limma *t*-test), 22 of them were found to be different between CR and CR-Leptin groups ($p \leq 0.010$; limma *t*-test) and simultaneously not statistically different between control and CR-Leptin pups, thus, we assumed that the expression levels of these genes were totally reverted by oral leptin treatment throughout lactation. The detailed list of these genes is shown in Table 3. Transcript levels of the remaining 196 genes, which appeared to be affected by moderate maternal calorie restriction during gestation, became partially reverted by leptin treatment throughout lactation (Supplementary Table 1). They include the set of genes whose expression profile differed significantly in CR *vs* control males ($p \leq 0.010$; limma *t*-test) but did not differ significantly in CR-Leptin *vs* CR and CR-Leptin *vs* control animals. Only 6 of the 224 genes whose expression levels were changed due to gestational calorie restriction (CR *vs* control males, $p \leq 0.010$; limma *t*-test) were not reverted by leptin treatment throughout lactation, as their expression levels did not differ between CR and CR-Leptin groups and were significantly different between control and CR-Leptin groups ($p \leq 0.010$; limma *t* test). These genes were the following: cardiomyopathy associated 5 *(Cmya5)*; defensin, alpha, 24 *(Defa24)*; desmoglein 2 *(Dsg2)*; secretory leukocyte peptidase inhibitor *(Slpi)*; tryptophan hydroxylase *2 (Tph2)*; and tripartite motif-containing 23 *(Trim23)*. They were related to the immune system, cell turnover, neural signaling and metabolism of protein processes.

Confirmation of microarray results in male animals by RT-qPCR and verification in females

To confirm gene array findings in male pups as well as to verify whether the described changes were also occurring in females, expression levels of a selected set of genes were analysed by RT-qPCR. The genes selected for analysis were those differentially expressed in CR animals vs their controls, and whose expression levels were totally reverted by leptin treatment throughout lactation (a number of 22 genes). Among those genes, the expression levels were found to be quantifiable by RT-qPCR for: collapsin response mediator protein 1 *(Crmp1)*; digestive organ expansion factor homolog (zebrafish) *(Diexf)*; fos-like antigen 1 *(Fosl1)*; galactosidase, alpha *(Gla)*; glutaminase *(Gls)*; low density lipoprotein receptor-related protein 11 *(Lrp11)*; polyamine oxidase (exo-N4-amino) *(Paox)*; ring finger protein 10 *(Rnf10)*; selenium binding protein 1

(Selenbp1); solute carrier family 7 (amino acid transporter light chain, L system), member 5 *(Slc7a5)*; thymosin beta 4, X-linked *(Tmsb4x)*; and ubiquitin associated and SH3 domain containing, B *(Ubash3b)*.

Table 3. Detailed list of genes which expression in PBMC samples of male rats at the age of 25 days was affected by gestational caloric restriction and totally reverted by oral leptin supplementation throughout lactation (CR≠controls; CR-Leptin≠CR; CR-Leptin=controls).

Biological process	Gene name	Gene symbol	Sequence ID	CR *vs* Controls		CR-Leptin *vs* CR	
				P	FC	*P*	FC
Cell communication	small cell adhesion glycoprotein	Smagp	NM_182817	0.000	-0.50	0.006	+0.41
Cell turnover	digestive organ expansion factor homolog (zebrafish)	Diexf	NM_001013986	0.002	+0.73	0.006	-0.66
	fos-like antigen 1	Fosl1	NM_012953	0.003	-0.64	0.009	+0.60
	selenium binding protein 1	Selenbp1	NM_080892	0.001	-0.93	0.007	+0.81
Cytoskeleton	myosin IIIB	Myo3b	NM_001191901	0.007	-0.42	0.010	+0.43
	thymosin beta 4, X-linked	Tmsb4x	NM_031136	0.010	+0.84	0.010	-0.91
Metabolism (carbohydrates)	galactosidase, alpha	Gla	NM_001108820	0.004	+0.88	0.004	-0.96
Metabolism (lipids)	arachidonate lipoxygenase 3	Aloxe3	NM_001105793	0.009	-0.54	0.005	+0.63
	butyryl Coenzyme A synthetase 1	Bucs1	NM_001108502	0.004	-0.47	0.004	+0.51
	low density lipoprotein receptor-related protein 11	Lrp11	NM_001106217	0.002	+0.70	0.001	-0.77
Metabolism proteins and polyamines)	glutaminase	Gls	NM_001109968	0.008	+0.74	0.007	-0.80
	polyamine oxidase (exo-N4-amino)	Paox	NM_001106311	0.008	+0.62	0.010	-0.64
	ubiquitin associated and SH3 domain containing, B	Ubash3b	NM_001191792	0.010	+0.45	0.000	-0.65
Neural signaling	cholinergic receptor, nicotinic, delta	Chrnd	NM_019298	0.006	-0.56	0.004	+0.62
	gamma-aminobutyric acid (GABA) A receptor, alpha 6	Gabra6	NM_021841	0.002	-1.00	0.004	+0.96
	glutamate receptor, metabotropic 6	Grm6	NM_022920	0.010	-0.66	0.004	+0.79
Nervous system	collapsin response mediator protein 1	Crmp1	NM_012932	0.004	+0.82	0.008	-0.78
	myelin transcription factor 1	Myt1	NM_001108615	0.007	-0.57	0.005	+0.64
	ring finger protein 10	Rnf10	NM_001011904	0.003	-1.22	0.010	+1.11
Signaling	calcium channel, voltage-dependent, L type, alpha 1C subunit	Cacna1c	ENSRNOT00000009343	0.006	-0.65	0.009	+0.66
Transport	FYVE and coiled-coil domain containing 1	Fyco1	NM_001106870	0.003	+0.57	0.010	-0.51
	solute carrier family 7 (amino acid transporter light chain, L system), member 5	Slc7a5	NM_017353	0.007	-0.87	0.000	+1.20

Controls: the offspring of rats with free access to standard chow diet; CR: the offspring of 20% calorie restricted dams during the first 12 days of pregnancy; CR-Leptin: CR rats daily supplemented with physiological doses of leptin throughout lactation. *p*-values (*P*) of microarray data (limma *t*-test) and fold change (FC) values of CR *vs* Controls and CR-Leptin *vs* CR comparisons are indicated; +, indicates upregulation; −, downregulation. Threshold of significance was set at $p \leq 0.010$.

The expression patterns of these genes in male and female animals are shown in Figure 2. RT-qPCR analysis fully confirmed most of the microarray data in male pups, and gene expression profile was largely similar in females. In detail, considering males and females, gestational calorie restriction resulted in higher expression levels of *Crmp1*, *Gla*, *Gls*, *Lrp11*, *Paox*, *Tmsb4x* and *Ubash3b;* in turn leptin treatment throughout lactation significantly diminished transcript levels of these genes to the control values

and hence reversed the effects observed in CR animals ($p<0.05$; LSD *post hoc* two-way ANOVA test).

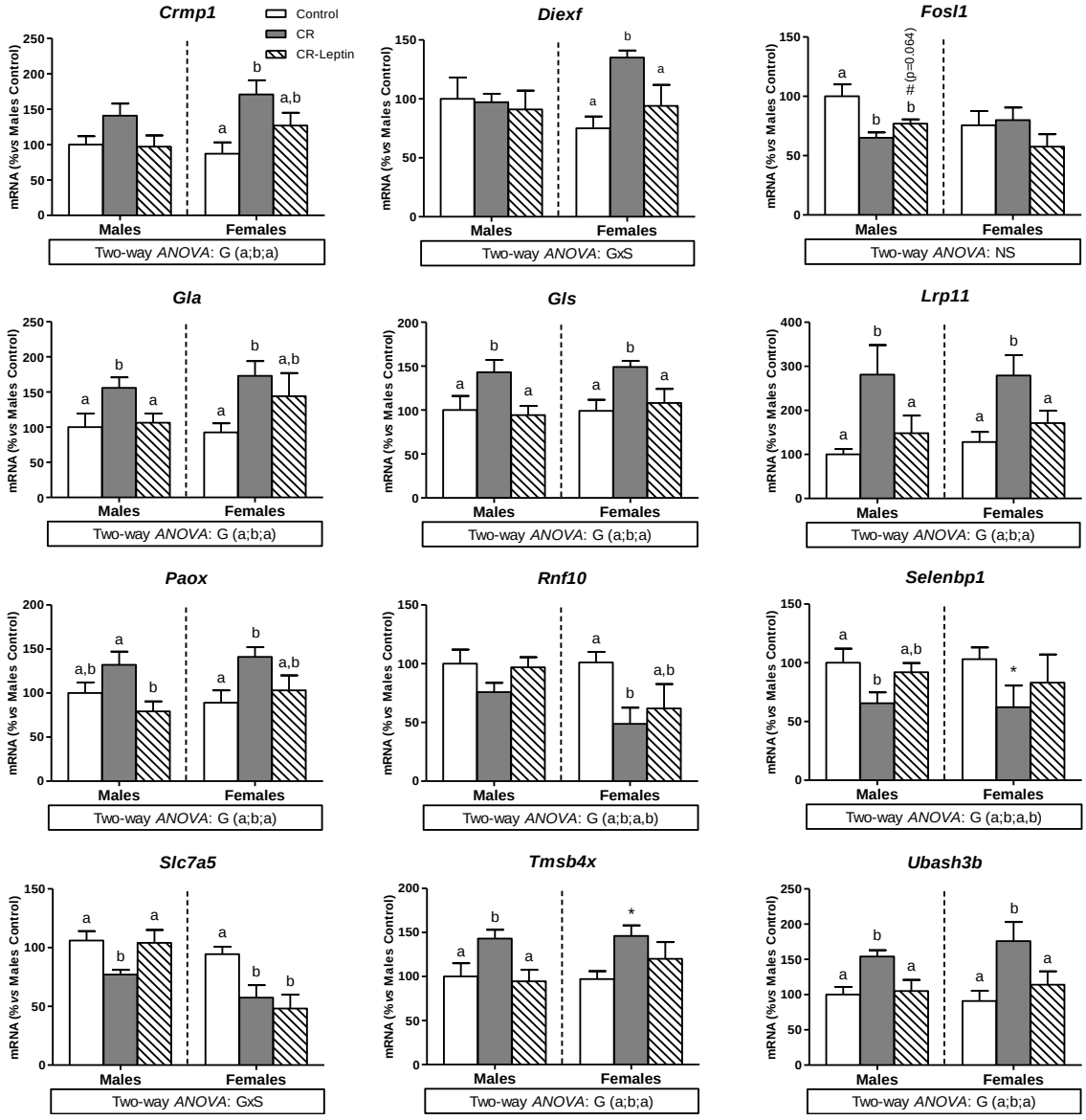

Figure 2. Q-PCR substantiation of microarray data of genes affected by gestational calorie restriction and totally reverted by oral leptin supplementation throughout lactation (CR≠controls; CR-Leptin≠CR; CR-Leptin=controls). To confirm and verify consistency of array findings, RT-qPCR was performed in PBMCs samples of both male and female offspring of control and calorie-restricted dams during gestation (CR) at the age of 25 days. mRNA levels were expressed as a percentage of the mean value of male controls. Data are mean ± S.E.M (n=7-10 animals/group). Statistical analysis was performed by considering males and females as a whole ($p\leq0.05$; two-way ANOVA), and separately for each sex, to study individual differences between groups (controls, CR, CR-Leptin) ($p\leq0.05$; one-way ANOVA). At any rate, data not sharing a common letter (a and b) are significantly different (a≠b) ($p<0.05$; LSD *post hoc* one-way and two-way ANOVA). Symbols: G, effect of group; GxS; interaction between the effect of group and the effect of sex ($p<0.05$; two-way ANOVA); *, CR *vs* Controls ($p<0.05$; Student's *t* test); #, CR-Leptin *vs* CR ($p<0.05$; Student's *t* test); NS, not statistically significant.

Differences concerning *Rnf10* and *Selenbp1* expression levels identified using the microarrays were partially confirmed by RT-qPCR analysis. Gestational calorie

restriction brought about down-regulation of mRNA levels of both genes in CR animals ($p \leq 0.05$; LSD *post hoc* two-way ANOVA). In turn, leptin supplementation throughout lactation partially normalized these values to those observed in controls ($p \leq 0.05$; LSD *post hoc*t wo-way ANOVA).

RT-qPCR analysis revealed that mRNA levels of *Diexf* and *Slc7a5* showed different expression pattern between sexes (interaction between group and sex; $p \leq 0.05$; two-way ANOVA). Leptin treatment throughout lactation resulted in total reversion of increased *Diexf* mRNA expression levels in CR females ($p \leq 0.05$; LSD *post hoc* one-way ANOVA), whereas no statistical differences were observed in males either due to gestational calorie restriction or due to leptin treatment throughout lactation. Regarding *Slc7a5* mRNA levels, as confirmed with both techniques used, decreased expression levels of this gene in CR animals were restored due to leptin treatment throughout lactation in males, but not in females ($p \leq 0.05$; LSD *post hoc* one-way ANOVA).

RT-qPCR analysis of *Fosl1* did not fully confirm the expression pattern of this gene observed with the microarray analysis. Although, its expression levels were confirmed to be diminished in CR males *vs* controls ($p \leq 0.05$; LSD *post hoc* one-way ANOVA), leptin treatment throughout lactation did not restore its control levels ($p \leq 0.05$; LSD *post hoc* one-way ANOVA). Nevertheless, single comparison between groups revealed that CR-Leptin males exhibited a trend to higher *Fosl1* mRNA levels relative to their CR counterparts ($p = 0.064$; Student's *t* test). Expression levels of *Fosl1* in females were not different between groups.

Discussion

One of the major challenges in developed societies is to implement therapeutic and, even more interestingly, preventive strategies, to fight the increased prevalence of obesity and its related metabolic disorders. In this regard, there is an increased need for identification of early biomarkers of propensity to these alterations (Musaad and Haynes 2007; Musaad and Haynes 2007). This could serve as a tool to improve the accuracy of disease diagnose and effectiveness of its prevention, as well as to monitor the efficacy of therapy used. Therefore, adding novel multimarkers beyond the traditional risk factors for obesity development may substantially improve early identification of susceptible individuals in order to implement effective therapeutic and/or preventive strategies. A growing number of studies exploring nutrigenomic-derived biomarkers

focus on blood cells as a surrogate tissue (Caimari et al. 2010; Oliver et al. 2013; de Mello et al. 2012; Sanchez et al. 2012; Priego et al. 2013), because they can be easily and repeatedly collected in humans, and hence may give a chance to apply results obtained from animal studies into humans. On the other hand, regarding obesity prevention, neonatal leptin treatment in rats has been found to prevent age- and high fat diet feeding-related overweight and other metabolic alterations (Pico et al. 2007; Sanchez et al. 2008; Priego et al. 2010). Moreover, the intake of appropriate amounts of leptin during lactation has been proposed as one of the strategies for reversing the effects of metabolic disorders induced as a consequence of developmental programming (Konieczna et al. 2013). Therefore, in this study we undertook these issues and attempted to identify early biomarkers that predict programmed susceptibility to obesity-related chronic diseases caused by moderate undernutrition during gestation and to monitor the response to neonatal leptin treatment using transcript-based biomarkers identified in PBMCs.

The outcomes of maternal undernutrition during gestation on the offspring have been broadly discussed in scientific literature. This prenatal condition exerts adverse health effects on the offspring leading to hyperphagia, hypertension and greater fat accumulation (Vickers et al. 2000; Palou et al. 2010), impaired insulin and leptin sensitivity (Palou et al. 2012), alterations in the structure and expression of neuropeptides regulating feeding behaviour in hypothalamus (Ikenasio-Thorpe et al. 2007; Garcia et al. 2010) as well as alterations in adipose tissue and stomach sympathetic innervations (Garcia et al. 2011; Garcia et al. 2013). Our study, for the first time, reveals, at the transcriptional level, the whole of the detrimental programming sequelae induced by mild gestational calorie restriction, by whole-genome transcriptome profiling of PBMCs of male offspring. The rational for performing microarray analysis only in males results from previous findings indicating that the overall detrimental effects of this prenatal condition were more marked in males than in females, particularly in terms of overweight and fat accumulation (Jones and Friedman 1982; Palou et al. 2010; Palou et al. 2012). In the present study we found that prenatal food restriction affected mRNA levels of 224 genes expressed in PBMCs. Induced changes in gene expression are mainly related to transcription and translation machinery, followed by genes related to immune system, which is not surprising as PBMCs are a subset of white blood cells. Gestational calorie restriction also affected

PBMCs expression of genes involved, among others, in signalling, cell turnover, metabolism, transport, cytoskeleton, sensory perception, nervous system and neural signaling. Hence, we identified a comprehensive set of easily assessed transcript-based biomarkers that reflect a wide system of biological processes and can improve prediction of the programmed susceptibility to obesity and other pathologies induced by fetal undernutrition. Although it is true that the presence of maternal undernutrition during gestation does not appear to account for the increasing prevalence of obesity in children and adults in developed societies nowadays, it might explain the increasing prevalence of obesity-related pathologies among people in developing countries where maternal malnutrition remains common (Rasmussen 2001). Moreover, fetal undernutrition may also arise as a result of deficiency caused by maternal failure of complete absorption of food components (McArdle and Ashworth 1999) and differences in seasonal food availability (Rasmussen 2001).

Interestingly, we also show here the overall effects of leptin supplementation throughout the suckling period in reverting gene expression alterations in PBMCs induced by maternal undernutrition during gestation. Neonatal leptin treatment normalized the expression of almost all of the genes whose expression was affected by calorie restriction during gestation in male offspring, with the exception of 6 genes. Among the 218 genes whose expression was normalized, 196 genes reverted partially and 22 were totally reverted. These findings support the relevance of the oral intake of leptin during lactation, which might be worth considering when searching for strategies to treat and/or prevent the programmed trend to obesity acquired by inadequate fetal nutrition, particularly in susceptible subgroups. Microarray findings in PBMC samples from male animals were substantiated by analyzing expression levels of a selection of genes by RT-qPCR. We focused on the 22 genes whose expression levels were completely normalized in leptin-treated CR male pups. mRNA expression levels of the same set of genes were also determined in PBMC samples of female animals to verify whether they behaviour was similar to that of males. Among the 22 genes analyzed, expression levels of several of them (*Crmp1*, *Gla*, *Gls*, *Lrp11*, *Paox*, *Tmsb4x* and *Ubash3b*) emerged as particularly promising to be used as biomarkers because qPCR analyses fully confirmed microarray data and showed similar behavior between males and females. In addition to the aforementioned genes, other genes such as *Rnf10*, *Selenbp1*, *Diexf* and *Slc7a5* may also be of interest. qPCR analysis of the expression of *Rnf10* and *Selenbp1* genes in both

genders showed similar patterns to those found for males with the microarray analysis, although decreased expression levels occurring in CR animals *vs* their controls were only partially reverted by leptin treatment during lactation. Sexual dimorphism was found concerning the expression pattern of the *Diexf* and *Slc7a5* genes in blood cells as an effect of treatments. The increased expression levels of the *Diexf* gene became totally reverted by leptin treatment, but only in the group of females, and no changes were found in males. Concerning *Slc7a5*, decreased expression levels of this gene occurring in CR animals were only normalized by leptin treatment during lactation in males, but not in females. Details on the proteins encoded by these genes and their potential functions are included as supplementary material (supplementary Table 2). Besides the annotated basal function of proteins encoded by these genes, some of them have been found to serve as a bridge for several key biological pathways and showed a significant association with some of the disease outcomes, although their specific function in PBMCs has not been generally explored.

Herewith, we identified a comprehensive set of easily assessed transcript-based biomarkers that reflect the reversion of a wide system of developmentally programmed biological processes induced by relative fetal undernutrition. Although the exact function of each of these genes in PBMCs remains to be determined, identification of these markers, indicators of efficacy of neonatal leptin treatment, supports evidence of the positive effects of leptin intake during the suckling period on later health programming.

Taken together, the results from this study show that leptin supplementation throughout lactation is able to revert the expression of most of the identified early potential biomarkers of programmed obesity risk and other metabolic alterations associated to poor nutrition during pregnancy. These findings support the relevance of the intake of leptin during lactation, a specific compound of maternal milk with beneficial health effects, which is not present in formula baby milks, and which might be worth considering when searching for strategies to treat and/or prevent the programmed trend to diseases acquired by inadequate fetal nutrition, particularly in susceptible subgroups. The use of the identified biomarkers may allow the identification and subsequent monitoring of individuals at early ages who are at higher risk to develop obesity and other pathologies and whose alterations can be reverted by the intake of breast milk with appropriate amounts of leptin during lactation.

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Supplementary table 1. Detailed list of genes whose expression levels in PBMC samples of male rats at the age of 25 days were affected by gestational caloric restriction and partially reverted by oral leptin supplementation throughout lactation (CR≠controls; CR-Leptin=CR; CR-Leptin=controls).

Biological Process	Gene Name	Gene Symbol	Sequence ID	CR vs Controls		CR-Leptin vs CR		CR-Leptin vs Controls	
				P	FC	P	FC	P	FC
Blood	elastase, neutrophil expressed	Elane	NM_001106767	0.010	-0.60	0.092	+0.40	0.355	-0.20
	pan hematopoietic expression	Phemx	XM_002725757	0.010	+0.60	0.057	-0.46	0.501	+0.15
	phospholipid scramblase 2	Plscr2	NM_001014094	0.009	+0.53	0.016	-0.52	0.941	+0.01
	protein tyrosine phosphatase, non-receptor type 9	Ptpn9	NM_001013040	0.009	+0.48	0.037	-0.40	0.639	+0.08
	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2	Serpinf2	NM_001011892	0.003	+0.48	0.032	-0.35	0.360	+0.13
Cell communication	CD99 molecule-like 2	Cd99l2	NM_134459	0.005	+0.95	0.170	-0.47	0.132	+0.48
	cadherin 19, type 2	Cdh19	NM_001009448	0.009	-0.82	0.154	+0.45	0.204	-0.37
	CKLF-like MARVEL transmembrane domain containing 3	Cmtm3	NM_001106164	0.007	+0.53	0.133	-0.30	0.206	+0.23
	catenin (cadherin associated protein), alpha-like 1	Ctnnal1	NM_001106649	0.008	+0.66	0.062	-0.47	0.426	+0.18
	integrin, alpha L	Itgal	NM_001033998	0.000	+0.71	0.033	-0.40	0.067	+0.31
	Pannexin 1	Panx1	NM_199397	0.006	+0.59	0.013	-0.55	0.855	+0.04
Cell turnover	B-cell CLL/lymphoma 2	Bcl2	NM_016993	0.002	+0.96	0.031	-0.69	0.351	+0.27
	bone morphogenetic protein 8a	Bmp8a	NM_001109432	0.007	-0.90	0.049	+0.67	0.464	-0.23
	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	Dyrk2	NM_001108100	0.008	+0.65	0.158	-0.35	0.195	+0.30
	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	Ets1	L20681	0.006	+1.05	0.154	-0.55	0.163	+0.50
	GIPC PDZ domain containing family, member 3	Gipc3	NM_001109282	0.008	-0.83	0.076	+0.57	0.355	-0.27
	headcase homolog (Drosophila)	Heca	NM_001107514	0.010	+0.57	0.094	-0.38	0.352	+0.19
	lymphoid enhancer binding factor 1	Lef1	NM_130429	0.007	+0.89	0.223	-0.40	0.114	+0.49
	mutS homolog 4 (E. coli)	Msh4	NM_001106477	0.002	-0.61	0.075	+0.35	0.141	-0.26
	metastasis suppressor 1	Mtss1	NM_001130563	0.004	+0.62	0.073	-0.39	0.238	+0.23
	myelocytomatosis oncogene	Myc	NM_012603	0.002	+1.04	0.173	-0.43	0.048	+0.61
	nucleosome assembly protein 1-like 1	Nap1l1	NM_053561	0.005	+0.68	0.022	-0.57	0.628	+0.11
	programmed cell death 4	Pdcd4	NM_022265	0.007	+0.59	0.084	-0.39	0.309	+0.21
	protamine 1	Prm1	NM_001002850	0.000	+0.56	0.241	-0.18	0.017	+0.37
	SNF2 histone linker PHD RING helicase	Shprh	NM_001107470	0.008	+0.55	0.292	-0.22	0.096	+0.33
	taxilin gamma	Txlng	ENSRNOT00000006843	0.004	+0.63	0.015	-0.56	0.722	+0.07
	zinc finger protein 259	Zfp259	NM_001137646	0.007	+0.47	0.125	-0.27	0.220	+0.20
Cytoskeleton	advillin	Avil	NM_024401	0.005	-0.55	0.029	+0.45	0.565	-0.10
	CD2-associated protein	Cd2ap	NM_181475	0.008	+0.57	0.034	-0.47	0.639	+0.09
	coronin, actin binding protein, 2B	Coro2b	ENSRNOT00000020951	0.007	+0.42	0.344	-0.15	0.065	+0.28
	Enah/Vasp-like	Evl	NM_024147	0.009	+0.92	0.083	-0.62	0.355	+0.30
	protein phosphatase 1, regulatory subunit 12C	Ppp1r12c	NM_001191946	0.004	+0.49	0.039	-0.36	0.400	+0.13
	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	Slc9a3r1	NM_021594	0.003	+0.51	0.335	-0.16	0.032	+0.35
	serine/threonine kinase 33	Stk33	ENSRNOT00000019597	0.008	-0.69	0.090	+0.45	0.322	-0.24

	WAS protein family homolog 2	Wash2	NM_001127390	0.003	+0.59	0.033	-0.43	0.399	+0.15
Epigenetic modification	DNA (cytosine-5-)-methyltransferase 3 alpha	Dnmt3a	NM_001003958	0.008	+0.73	0.076	-0.50	0.366	+0.23
	lysine (K)-specific demethylase 6B	Kdm6b	NM_001108829	0.009	-0.48	0.055	+0.37	0.502	-0.12
	sirtuin 5	Sirt5	NM_001004256	0.010	+0.60	0.060	-0.45	0.496	+0.15
	SET and MYND domain containing 2	Smyd2	NM_206851	0.008	+0.70	0.156	-0.38	0.191	+0.32
Immune system	B-cell receptor-associated protein 31	Bcap31	NM_001004224	0.003	+0.71	0.024	-0.56	0.487	+0.15
	cathelicidin antimicrobial peptide	Camp	CB577971	0.009	-0.54	0.121	+0.33	0.273	-0.21
	Cd2 molecule	Cd2	NM_012830	0.002	+0.78	0.062	-0.48	0.202	+0.30
	Cd247 molecule	Cd247	NM_170789	0.003	+0.75	0.160	-0.35	0.096	+0.40
	CD40 ligand	Cd40lg	NM_053353	0.003	+0.99	0.072	-0.60	0.196	+0.39
	cytotoxic T lymphocyte-associated protein 2 alpha	Ctla2a	NM_001109115	0.001	+0.88	0.026	-0.60	0.243	+0.28
	IgA Fc receptor	Fcar	NM_201992	0.006	-0.84	0.200	+0.39	0.110	-0.45
	guanine nucleotide binding protein-like 1	Gnl1	NM_212500	0.006	+0.60	0.034	-0.47	0.529	+0.13
	inducible T-cell co-stimulator	Icos	NM_022610	0.007	+0.70	0.054	-0.52	0.435	+0.19
	interferon-induced protein 44-like	Ifi44l	XM_227820	0.005	+1.01	0.089	-0.61	0.223	+0.40
	interleukin 10	Il10	NM_012854	0.002	-0.59	0.224	+0.23	0.042	-0.36
	interleukin 15	Il15	NM_013129	0.006	+0.65	0.135	-0.35	0.171	+0.30
	IL2-inducible T-cell kinase	Itk	NM_001108825	0.009	+1.07	0.213	-0.51	0.143	+0.56
	linker for activation of T cells	Lat	NM_030853	0.005	+0.96	0.078	-0.61	0.266	+0.35
	myxovirus (influenza virus) resistance 1	Mx1	NM_173096	0.010	+0.89	0.227	-0.42	0.146	+0.47
	peptidoglycan recognition protein 4	Pglyrp4	NM_001191708	0.008	-0.60	0.082	+0.40	0.354	-0.19
	RT1 class I, locus A3	RT1-A3	NM_001008830	0.004	+1.06	0.176	-0.49	0.093	+0.57
	RT1 class Ib, locus EC2	RT1-EC2	M10094	0.004	+1.22	0.183	-0.55	0.088	+0.67
	src kinase associated phosphoprotein 1	Skap1	NM_173311	0.010	+0.94	0.178	-0.50	0.195	+0.44
	T-cell receptor beta chain	Tcrb	BC091428	0.010	+0.77	0.222	-0.36	0.152	+0.40
	unc-13 homolog D (C. elegans)	Unc13d	NM_138844	0.009	+0.54	0.033	-0.45	0.658	+0.08
	zeta-chain (TCR) associated protein kinase	Zap70	NM_001012002	0.009	+1.08	0.144	-0.62	0.234	+0.47
Metabolism (carbohydrates)	glycosyltransferase 1 domain containing 1	Glt1d1	ENSRNOT00000064526	0.004	-0.71	0.023	+0.57	0.536	-0.14
	glucosamine-phosphate N-acetyltransferase 1	Gnpnat1	NM_001134757	0.010	+0.60	0.062	-0.45	0.488	+0.15
	heparanase	Hpse	NM_022605	0.005	+0.82	0.036	-0.63	0.484	+0.19
	phosphofructokinase, platelet	Pfkp	L25387	0.005	+0.62	0.087	-0.38	0.241	+0.24
Metabolism (central)	iron responsive element binding protein 2	Ireb2	NM_022863	0.010	+0.66	0.022	-0.61	0.843	+0.05
	iron-sulfur cluster assembly 1 homolog (S. cerevisiae)	Isca1	NM_181626	0.010	-0.74	0.020	+0.71	0.917	-0.03
	methylenetetrahydrofolate reductase (NAD(P)H)	Mthfr	ENSRNOT00000011384	0.010	-0.57	0.086	+0.39	0.389	-0.18
Metabolism (lipids)	acid phosphatase 6, lysophosphatidic	Acp6	NM_001031645	0.002	+0.75	0.101	-0.40	0.122	+0.35
	choline phosphotransferase 1	Chpt1	NM_001007750	0.008	+0.67	0.022	-0.60	0.763	+0.07
	emopamil binding protein-like	Ebpl	NM_001108381	0.003	+0.77	0.106	-0.42	0.150	+0.35
	fatty acid amide hydrolase	Faah	NM_024132	0.007	+0.79	0.187	-0.39	0.137	+0.41
	low density lipoprotein receptor adaptor protein 1	Ldlrap1	NM_001109271	0.002	+0.81	0.043	-0.54	0.258	+0.27
	oxysterol binding protein-like 1A	Osbpl1a	NM_172023	0.002	+0.75	0.037	-0.52	0.304	+0.23
	3-oxoacyl-ACP synthase, mitochondrial	Oxsm	NM_001100508	0.010	+0.50	0.129	-0.30	0.263	+0.20
	phosphatidylinositol glycan anchor biosynthesis, class L	Pigl	ENSRNOT00000004113	0.007	+0.62	0.053	-0.46	0.445	+0.16
	phospholipase A2, group XIIA	Pla2g12a	NM_001108565	0.004	+0.77	0.042	-0.54	0.358	+0.22
	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1	St8sia1	NM_012813	0.008	+0.91	0.053	-0.68	0.472	+0.23
Metabolism	adenylosuccinate lyase	Adsl	NM_001130503	0.010	+0.48	0.090	-0.33	0.368	+0.16

(nucleotides)	phosphoribosylglycinamide formyltransferase	Gart	BC087644	0.010	+0.48	0.067	-0.35	0.467	+0.13
	5'-nucleotidase, cytosolic III-like	Nt5c3l	NM_001007723	0.006	+0.76	0.272	-0.30	0.084	+0.46
	phosphoribosyl pyrophosphate synthetase-associated protein 1	Prpsap1	NM_022545	0.006	+0.57	0.014	-0.53	0.833	+0.04
Metabolism (proteins and polyamines)	anterior pharynx defective 1 homolog A (C. elegans)	Aph1a	NM_001014255	0.008	-0.43	0.063	+0.31	0.429	-0.12
	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 8	B3gnt8	NM_001107492	0.006	-0.61	0.026	+0.50	0.614	-0.10
	core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1	C1galt1	NM_022950	0.009	+0.69	0.066	-0.49	0.428	+0.19
	calreticulin	Calr	NM_022399	0.004	-0.64	0.029	+0.50	0.490	-0.14
	casein kinase 1, gamma 2	Csnk1g2	NM_023102	0.009	+0.56	0.081	-0.38	0.373	+0.18
	cathepsin W	Ctsw	NM_001024242	0.009	+0.72	0.147	-0.40	0.218	+0.32
	leucine carboxyl methyltransferase 1	Lcmt1	NM_199405	0.005	+0.58	0.151	-0.30	0.149	+0.28
	protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 2	Pcmtd2	NM_001107810	0.000	+0.72	0.158	-0.27	0.018	+0.45
	protein arginine methyltransferase 3	Prmt3	NM_053557	0.003	+0.74	0.052	-0.49	0.281	+0.25
	proteinase 3	Prtn3	NM_001024264	0.006	-0.69	0.023	+0.59	0.657	-0.10
	ring finger protein 125	Rnf125	NM_001108424	0.009	+0.94	0.150	-0.52	0.216	+0.42
	serine (or cysteine) peptidase inhibitor, clade B, member 6b	Serpinb6b	NM_001012214	0.005	+0.86	0.072	-0.55	0.268	+0.31
	transglutaminase 1, K polypeptide	Tgm1	NM_031659	0.003	-0.87	0.432	+0.23	0.024	-0.64
	transglutaminase 6	Tgm6	ENSRNOT00000009097	0.009	-0.49	0.047	+0.38	0.528	-0.11
	ubiquitin-like modifier activating enzyme 5	Uba5	NM_001009669	0.004	+0.91	0.084	-0.54	0.199	+0.37
	URI1, prefoldin-like chaperone	Uri1	NM_001107507	0.002	+0.95	0.014	-0.78	0.532	+0.17
Metabolism (redox)	prolyl 4-hydroxylase, alpha polypeptide II	P4ha2	NM_001108275	0.004	-0.79	0.520	+0.18	0.022	-0.62
	STEAP family member 4	Steap4	NM_001044265	0.006	-0.51	0.118	+0.29	0.214	-0.22
	thioredoxin interacting protein	Txnip	NM_001008767	0.001	+0.91	0.098	-0.46	0.079	+0.46
Neural signaling	4-aminobutyrate aminotransferase	Abat	NM_031003	0.006	+0.69	0.150	-0.36	0.159	+0.33
	hippocalcin-like 1	Hpcal1	NM_017356	0.009	+0.45	0.090	-0.30	0.331	+0.16
	potassium voltage-gated channel, KQT-like subfamily, member 4	Kcnq4	AF249748	0.010	-0.69	0.724	+0.09	0.022	-0.60
	protein phosphatase 1, regulatory subunit 9A	Ppp1r9a	NM_053473	0.007	-0.60	0.088	+0.38	0.287	-0.22
Nervous system	adhesion molecule with Ig like domain 1	Amigo1	BC167749	0.005	+0.87	0.062	-0.59	0.336	+0.28
	churchill domain containing 1	Churc1	NM_001106741	0.009	-0.52	0.306	+0.20	0.099	-0.31
	membrane protein, palmitoylated 5 (MAGUK p55 subfamily member 5)	Mpp5	NM_001108034	0.004	+0.73	0.035	-0.54	0.411	+0.19
	platelet-activating factor acetylhydrolase, isoform 1b, subunit 3	Pafah1b3	NM_053654	0.009	+0.54	0.080	-0.37	0.366	+0.17
	progesterone receptor membrane component 1	Pgrmc1	NM_021766	0.006	+0.80	0.029	-0.66	0.592	+0.14
	staufen, RNA binding protein, homolog 2 (Drosophila)	Stau2	NM_001007149	0.004	+0.77	0.019	-0.65	0.608	+0.12
Others	angiomotin like 2	Amotl2	NM_031717	0.005	-0.61	0.052	+0.43	0.356	-0.18
	ADP-ribosylation factor-like 5A	Arl5a	ENSRNOT00000009181	0.005	+0.55	0.103	-0.32	0.202	+0.23
	glutathione S-transferase pi 1	Gstp1	NM_012577	0.007	+0.59	0.044	-0.45	0.477	+0.14
	mucin 5B, oligomeric mucus/gel-forming	Muc5b	ENSRNOT00000028967	0.009	-0.93	0.024	+0.84	0.794	-0.09
	nephrosis 1, congenital, Finnish type	Nphs1	NM_022628	0.006	-0.68	0.202	+0.31	0.118	-0.36
	SPO11 meiotic protein	Spo11	NM_001108964	0.006	+0.62	0.101	-0.37	0.234	+0.25

	covalently bound to DSB homolog (S. cerevisiae)								
	zona pellucida binding protein 2	Zpbp2	NM_001007011	0.005	+0.60	0.079	-0.38	0.253	+0.22
Sensory perception	cyclic nucleotide gated channel alpha 1	Cnga1	NM_053497	0.006	+0.72	0.138	-0.39	0.168	+0.33
	olfactory receptor 107	Olr107	NM_001000148	0.006	-0.64	0.037	+0.50	0.490	-0.15
	olfactory receptor 1075	Olr1075	NM_001000421	0.001	-0.95	0.015	+0.72	0.366	-0.23
	olfactory receptor 1394	Olr1394	NM_001001091	0.002	+0.72	0.027	-0.53	0.373	+0.19
	olfactory receptor 1481	Olr1481	NM_001000527	0.008	-0.43	0.158	+0.23	0.194	-0.20
	olfactory receptor 1500	Olr1500	NM_001000942	0.001	+0.71	0.347	-0.19	0.012	+0.51
	olfactory receptor 1602	Olr1602	NM_001000909	0.004	-0.71	0.274	+0.26	0.055	-0.45
	olfactory receptor 239	Olr239	NM_001000211	0.005	-0.52	0.557	+0.11	0.020	-0.42
	olfactory receptor 439	Olr439	NM_001000281	0.009	+0.74	0.028	-0.64	0.722	+0.09
	olfactory receptor 500	Olr500	NM_001000680	0.006	+0.58	0.493	-0.14	0.032	+0.44
Signaling	axin 2	Axin2	NM_024355	0.009	+0.65	0.143	-0.37	0.234	+0.28
	calcium/calmodulin-dependent protein kinase IV	Camk4	NM_012727	0.009	+0.81	0.049	-0.63	0.539	+0.18
	CD38 molecule	Cd38	NM_013127	0.006	+0.81	0.072	-0.54	0.314	+0.27
	COMM domain containing 7	Commd7	NM_001030029	0.008	+0.63	0.149	-0.34	0.192	+0.29
	diacylglycerol kinase, alpha	Dgka	NM_080787	0.010	+0.92	0.168	-0.49	0.205	+0.42
	GDP dissociation inhibitor 1	Gdi1	NM_017088	0.010	+0.47	0.035	-0.40	0.689	+0.07
	interleukin 21 receptor	Il21r	NM_001012469	0.010	+0.78	0.170	-0.42	0.208	+0.36
	interleukin 7 receptor	Il7r	NM_001106418	0.008	+0.84	0.039	-0.69	0.594	+0.16
	myelin protein zero-like 1	Mpzl1	NM_001007728	0.008	-0.53	0.121	+0.32	0.249	-0.22
	nemo like kinase	Nlk	NM_001191924	0.008	+0.57	0.017	-0.54	0.884	+0.03
	natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)	Npr1	NM_012613	0.005	-0.40	0.735	+0.05	0.012	-0.35
	progestin and adipoQ receptor family member V	Paqr5	NM_001014092	0.007	+0.97	0.154	-0.51	0.177	+0.46
	protein kinase (cAMP-dependent, catalytic) inhibitor alpha	Pkia	FQ211701	0.003	+0.92	0.046	-0.62	0.286	+0.30
	phospholipase C, gamma 1	Plcg1	NM_013187	0.008	+0.57	0.186	-0.29	0.159	+0.29
	protein kinase C, theta	Prkcq	ENSRNOT00000025901	0.007	+0.95	0.097	-0.59	0.274	+0.36
	RAS p21 protein activator 4	Rasa4	XM_002724808	0.006	+0.53	0.241	-0.23	0.098	+0.30
	ret proto-oncogene	Ret	NM_001110099	0.008	+0.90	0.168	-0.47	0.173	+0.43
	regulator of G-protein signaling 1	Rgs1	NM_019336	0.007	+1.73	0.076	-1.16	0.333	+0.58
	signal transducer and activator of transcription 4	Stat4	NM_001012226	0.007	+0.80	0.225	-0.36	0.113	+0.44
	serine/threonine kinase 4	Stk4	NM_001107800	0.002	+0.74	0.023	-0.53	0.324	+0.21
	tetraspanin 6	Tspan6	NM_001100672	0.008	+0.53	0.101	-0.33	0.279	+0.20
	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Ywhaz	NM_013011	0.008	+0.62	0.019	-0.57	0.822	+0.05
	Rho guanine nucleotide exchange factor (GEF) 1	Arhgef1	NM_021694	0.006	+0.63	0.103	-0.38	0.243	+0.25
Transcription / translation machinery	cirrhosis, autosomal recessive 1A (cirhin)	Cirh1a	NM_001009640	0.002	+0.67	0.066	-0.40	0.183	+0.27
	CREB regulated transcription coactivator 1	Crtc1	NM_001047115	0.000	-0.89	0.121	+0.36	0.019	-0.53
	DEAD (Asp-Glu-Ala-Asp) box polypeptide 50	Ddx50	NM_001013198	0.005	+0.61	0.049	-0.43	0.367	+0.18
	DEAH (Asp-Glu-Ala-His) box polypeptide 32	Dhx32	NM_001130039	0.005	+0.49	0.016	-0.44	0.714	+0.06
	DMRT-like family C1a	Dmrtc1a	NM_001025288	0.005	-0.75	0.037	+0.58	0.481	-0.17
	eukaryotic translation elongation factor 2	Eef2	NM_017245	0.006	+0.58	0.024	-0.49	0.633	+0.09
	eukaryotic translation initiation factor 3, subunit E	Eif3e	NM_001011990	0.010	+0.56	0.044	-0.45	0.606	+0.10
	FtsJ methyltransferase domain containing 1	Ftsjd1	NM_001106186	0.007	+0.54	0.025	-0.46	0.666	+0.08
	GAR1 ribonucleoprotein homolog (yeast)	Gar1	NM_001024306	0.006	+0.78	0.021	-0.69	0.716	+0.09
	Meis homeobox 3	Meis3	NM_001108472	0.003	+0.64	0.068	-0.39	0.198	+0.25
	nuclear receptor co-repressor 1	Ncor1	XM_001077495	0.005	+0.54	0.024	-0.45	0.629	+0.09
	NIN1/RPN12 binding protein 1 homolog (S. cerevisiae)	Nob1	NM_199086	0.009	+0.55	0.048	-0.43	0.526	+0.12

	nucleolar complex associated 2 homolog (S. cerevisiae)	Noc2l	NM_001033897	0.008	+0.61	0.033	-0.52	0.646	+0.10
	nucleolar protein 9	Nol9	ENSRNOT00000013535	0.010	+0.54	0.064	-0.40	0.478	+0.14
	similar to phosphoseryl-tRNA kinase	Pstk	ENSRNOT00000027967	0.009	+0.59	0.083	-0.40	0.359	+0.19
	RNA binding motif (RNP1, RRM) protein 3	Rbm3	NM_053696	0.008	+0.46	0.173	-0.24	0.170	+0.22
	similar to 40S ribosomal protein S19	RGD1559724	ENSRNOT00000047882	0.010	+0.75	0.077	-0.53	0.406	+0.22
	similar to phosphoseryl-tRNA kinase	RGD1564300	BC168162	0.010	+0.70	0.052	-0.54	0.533	+0.16
	ribosomal protein S11	Rps11	NM_031110	0.006	+0.75	0.033	-0.61	0.569	+0.14
	ribosomal protein S19	Rps19	NM_001037346	0.003	+0.78	0.035	-0.57	0.386	+0.21
	SATB homeobox 1	Satb1	NM_001012129	0.004	+0.90	0.112	-0.50	0.176	+0.40
	T-box 3	Tbx3	NM_181638	0.010	-0.52	0.107	+0.33	0.309	-0.19
	transcription factor 12	Tcf12	NM_013176	0.008	+0.80	0.031	-0.67	0.649	+0.13
	tRNA methyltransferase 11 homolog (S. cerevisiae)	Trmt11	ENSRNOT00000019436	0.006	+0.55	0.046	-0.41	0.436	+0.14
	TSR2, 20S rRNA accumulation, homolog (S. cerevisiae)	Tsr2	NM_001115027	0.009	+0.61	0.078	-0.42	0.380	+0.19
	UPF3 regulator of nonsense transcripts homolog B (yeast)	Upf3b	NM_001135873	0.009	+0.46	0.085	-0.31	0.354	+0.15
	WD repeat domain 77	Wdr77	NM_001008771	0.006	+0.63	0.119	-0.36	0.199	+0.27
	zinc finger CCCH-type containing 15	Zc3h15	NM_001010963	0.002	+0.58	0.059	-0.35	0.179	+0.23
	zinc finger protein 503	Zfp503	NM_001107250	0.002	-0.52	0.031	+0.37	0.316	-0.15
	zinc finger protein 638	Zfp638	NM_001107868	0.006	+0.45	0.019	-0.40	0.722	+0.05
	zinc finger protein 709	Zfp709	NM_153731	0.007	+0.51	0.119	-0.30	0.234	+0.21
	zinc finger, HIT-type containing 6	Znhit6	NM_001106203	0.010	+0.69	0.070	-0.50	0.432	+0.20
	B-cell receptor-associated protein 29	Bcap29	NM_001006980	0.001	+0.76	0.057	-0.45	0.144	+0.32
Transport	cytohesin 1	Cyth1	NM_053910	0.005	+0.70	0.112	-0.39	0.186	+0.30
	potassium large conductance calcium-activated channel, subfamily M, beta member 4	Kcnmb4	NM_023960	0.007	+0.81	0.146	-0.44	0.190	+0.37
	NIPA-like domain containing 1	Nipal1	NM_001106003	0.007	+0.52	0.107	-0.31	0.254	+0.20
	phosphofurin acidic cluster sorting protein 1	Pacs1	NM_134406	0.004	+0.60	0.105	-0.34	0.175	+0.26
	sodium channel, nonvoltage-gated 1, beta	Scnn1b	NM_012648	0.009	+0.49	0.131	-0.28	0.242	+0.20
	solute carrier family 12 (potassium/chloride transporters), member 7	Slc12a7	NM_001013144	0.010	+0.56	0.096	-0.37	0.349	+0.19
	solute carrier family 16, member 8 (monocarboxylic acid transporter 3)	Slc16a8	NM_031744	0.005	-0.51	0.081	+0.32	0.267	-0.19
	solute carrier family 18 (vesicular monoamine), member 2	Slc18a2	NM_013031	0.010	+0.72	0.122	-0.44	0.275	+0.28
	solute carrier family 34 (sodium phosphate), member 3	Slc34a3	NM_139338	0.007	+0.59	0.352	-0.20	0.062	+0.39
	solute carrier family 9 (sodium/hydrogen exchanger), isoform 9	Slc9a9	XM_001064905	0.010	+0.56	0.100	-0.36	0.331	+0.20
	TSC22 domain family, member 3	Tsc22d3	NM_031345	0.002	+0.86	0.138	-0.39	0.066	+0.46
	VAMP (vesicle-associated membrane protein)-associated protein B and C	Vapb	NM_021847	0.004	+0.51	0.011	-0.47	0.805	+0.04

Controls: the offspring of rats with free access to standard chow diet; CR: the offspring of 20% calorie restricted dams during the first 12 days of pregnancy; CR-Leptin: CR rats daily supplemented with physiological doses of leptin throughout lactation. *p*-values (*P)* of microarray data (limma *t*-test) and fold change (FC) values of CR *vs* Controls, CR-Leptin vs CR and CR-Leptin *vs* Controls comparisons are indicated; +, indicates upregulation; –, downregulation. Threshold of significance was set at $p \leq 0.010$.

Supplementary table 2. Function of selected genes from the microarray analysis of PBMC samples of male rats at the age of 25 days which expression was affected by gestational caloric restriction and became totally reverted by oral leptin supplementation throughout lactation.

Gene symbol	Gene name	Protein codified	Function
Crmp1	Collapsin response mediator protein 1	CRMP1	Member of the Collapsin response mediator protein (CRMP) family of proteins highly expressed in developing and adult nervous systems and implicated in axon guidance and outgrowth (1). *Crmp1* has been characterized as a potential invasion-suppressor gene (2).
Diexf	Digestive Organ Expansion Factor Homolog (Zebrafish)	DIEXF	In zebrafish, acts as a pan-endoderm factor to coordinate the expansion growth of the entire digestive system. A loss-of-function mutation in Zebrafish of *Diexf* gene results in compromised organ growth (3).
Gla	Galactosidase, alpha	GLA	Homodimeric glycoprotein that hydrolyses the terminal alpha-galactosyl moieties from glycolipids and glycoproteins. Defectsin GLA activity, which is characteristic of the Fabry disease, leads to the systemic accumulation of neutral glycosphingolipids with terminal alpha-galactosyl moieties (4).
Gls	Glutaminase	GLS	K-type mitochondrial glutaminase, a protein that catalyzes the hydrolysis of glutamine to glutamate and ammonia. This protein is primarily expressed in the brain and kidney and plays an essential role in generating energy for metabolism, synthesizing the brain neurotransmitter glutamate and maintaining acid-base balance in the kidney (5).
Lrp11	Low density lipoprotein receptor-related protein 11	LRP11	Member of the LDL receptor family, a class of structurally closely related cell surface receptors fulfilling diverse functions in different tissues, which has been recently positioned as one of the key players in Alzheimer disease research (6).
Paox	Polyamine oxidase (exo-N4-amino)	PAO	Flavoenzyme that catalyzes the oxidation of N(1)-acetylspermine to spermidine and hence is involved in the polyamine back-conversion, thus playing an important role in the regulation of polyamine intracellular concentration. However, PAO activity results in production of ammonia, the corresponding amino aldehydes, and hydrogen peroxide. Malondialdehyde (MDA) and acrolein, potentially toxic agents, which induce oxidative stress in mammalian cells, are spontaneously formed from aminoaldehydes. Thus, resulting products of PAO activity have the potential to produce disease states. Increased PAO activity has been found in type 1 diabetic children, associated to increased blood HbA(1C) and MDA levels, demonstrating that increased plasma PAO activity may participate in these circumstances (7). Activation of PAO has also been associated to macrophage apoptosis due to hydrogen peroxide release and mitochondrial membrane depolarization, contributing to deficiencies in host defence in diseases such as H. pylori infection (8).
Rnf10	Ring finger protein 10	RNF10	The protein contains a ring finger motif, which is known to be involved in protein-protein interactions. The specific function of this protein has not yet been determined, but recent findings pinpoint its critical role in myelin formation and neuronal differentiation (9;10).
Selenbp1	Selenium binding protein 1	SELENBP1	Member of selenoproteins family. These proteins bind selenium covalently and mediate the intracellular transport of selenium (11). A deficiency of dietary selenium is associated with an increased

			incidence of epithelial cancers including lung, liver, colorectal, and prostate cancer (12). Selenium exerts its anticarcinogenic effects mainly through selenoproteins, and expression of SELENBP1 has been found to be reduced markedly in multiple epithelial cancers compared with their corresponding normal tissues. The exact function of SELENBP1 is not known, although, a possible link to malignancies associated with selenium deficiencies has been suggested (13;14).
Slc7a5	Solute carrier family 7 (amino acid transporter light chain, L system), member 5	SLC7A5, LAT1	It is involved in the sodium-independent cellular transport of amino acids with large neutral amino acid side chains (15). Increased expression levels of *Slc7a5* gene were positively correlated with increased biological aggressiveness and higher mortality in a range of human cancers (16). Expression levels of this gene may be nutritionally modulated, since decreased expression levels were described in brain tissues of pigs fed low dietary protein (17).
Tmsb4x	Thymosin beta 4, X-linked	Tß4	The major actin-sequestering protein in all eukaryotic cells and a potent regulator of actin polymerization in mammals (18). Numerous studies have identified a range of functions and activities for Tß4 important for wound healing and repair and tissue regeneration (18). Tß4 also appears to be involved in the regulation of the development and regeneration of the nervous system, as a novel neurotrophic signal (19). It is highly expressed in most neural cell types of the developing brain, and has also been found to be abundant in the injured/regenerating axons, suggesting that the upregulation of Tß4 is related to the axonal sprouting and neuronal regeneration. On the other hand, up-regulation of *Tmsb4x* gene has been discovered in a wide variety of human carcinomas and has been proposed as a key event in the acquisition of growth advantages as well as invasive phenotypes in human colorectal carcinomas (20).
Ubash3b	Ubiquitin associated and SH3 domain containing, B	UBASH3B, STS-1	The protein exhibits tyrosine phosphatase activity toward several substrates. This protein contains an ubiquitin associated domain at the N-terminus. Proteins containing ubiquitin-binding domains (UBDs) interact with ubiquitinated targets and regulate diverse biological processes, including endocytosis, signal transduction, transcription and DNA repair (21). Overexpression of this gene has been identified in aggressive cancers like triple-negative breast cancer, and promotes invasion and metastasis (22).

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MANUSCRIPT 6

Early biomarkers identified in a rat model of a healthier phenotype based on early postnatal dietary intervention may predict the response to an obesogenic environment in adulthood

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Title page

Title: Early biomarkers identified in a rat model of a healthier phenotype based on early postnatal dietary intervention may predict the response to an obesogenic environment in adulthood

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Conflict of interest: None

Abstract

Moderate maternal calorie restriction during lactation in rats provides certain protection against obesity in adult offspring. Hence, we used this model with 20% calorie restriction to identify early changes at gene expression level in key tissues involved in energy homeostasis, as well as to assess whether they are maintained in adulthood, to consider them as potential biomarkers of metabolic health. Offspring of control and 20% calorie restricted dams during lactation (CRL) were followed. Animals were studied at weaning and at 6 months old under normal-fat (NF) diet and after being moved to a high-fat (HF) diet for the last 2 months. Adult CRL animals showed lower body weight, decreased hepatic lipids, and improved circulating parameters versus controls. At weaning, CRL pups, in retroperitoneal white adipose tissue (rWAT), displayed lower mRNA levels of lipogenesis-related genes and higher mRNA levels of genes related with lipolysis and insulin signalling versus controls. CRL animals also showed lower hepatic mRNA levels of the lipogenesis-related gene Srebp1c and higher mRNA levels of Cpt1a, Atgl and Obrb. Some of these changes were sustained in adulthood under HF-diet, and mRNA levels of Irs1 (rWAT) and of Obrb and Srebp1c (liver) in adult animals correlated with hepatic lipids and circulating parameters. In conclusion, the protective effects of moderate calorie restriction during lactation on offspring metabolic health are reflected in early changes at gene expression level in key tissues. Among them, transcript levels of Irs1 (rWAT) and of Obrb and Srebp1c (liver) emerge as particularly interesting as potential transcript-based biomarkers of metabolic health.

Keywords: adipose tissue; calorie restriction; early markers; lactation; liver; obesity

Introduction

Obesity is a worldwide epidemic and a key risk factor in the development of insulin resistance, type 2 diabetes, hypertension and cardiovascular disease (Taylor and Poston 2007). Its prevalence is mainly associated to unhealthy habits, such as a sedentary lifestyle and excessive calorie intake, together with genetic factors. However, emerging evidence in humans and animal models indicates that early life nutrition may also play an important role programming the risk of obesity and other features of the metabolic syndrome in adult life (McMillen et al. 2008; Pico et al. 2012). In this regard, pregnancy and lactation are revealed as critical periods, where maternal diabetes, overnutrition or undernutrition may lead to permanent adaptations with lasting effects on metabolic mechanism in the offspring, thereby changing the propensity to obesity and related metabolic alterations in adult life (McMillen et al. 2005). In this sense, the undernourishment produced by protein restriction or severe calorie restriction (up to 50%) of lactating dams, which has been related with lower body weight of offspring, affects the normal development of offspring (de Moura et al. 2007; Fagundes et al. 2007). However, we have previously described that moderate maternal calorie restriction (30%) during the suckling period results in lower body weight and fat content of the offspring, without affecting body length (Palou et al. 2010a). Moreover, these animals are protected against the detrimental effects of a high-fat (HF) diet feeding in adulthood; they show changes at the gene expression level in key tissues involved in energy balance, related with higher leptin and insulin signaling, which could explain the improved capacity of these animals to handle and store the excess of fuel from the diet, thus providing a better adaptation to the challenge of HF-diet (Palou et al. 2011). Therefore, the exploration of mechanisms by which early maternal diet could program offspring for lower susceptibility to obesity in adulthood becomes of interest. This model may also be helpful to develop preventive strategies and identify early biomarkers of improved metabolic health.

Besides, although direct animal-to-human extrapolation is complex, regarding the beneficial effects of moderate calorie restriction during lactation on offspring metabolic health in rats, it would be of interest to consider its potential application in humans. However, studies on early postnatal undernutrition in lactating women are fewer due to ethical implications. Epidemiologic reports, and especially those related to reduction of maternal food intake during lactation, have described the effects on infant body weight gain or on maternal milk production depending on the severity of the restriction (Dewey 1998; Dusdieker et al. 1994). Other studies carried out in baboons, have shown no significant effect on milk output with a

moderate calorie restriction of 20%, but milk output was significantly lower in those with 40% calorie restriction (Roberts et al. 1985). Therefore, although 30% calorie restriction during lactation in rats has shown benefits in the offspring preventing later obesity (Palou et al. 2010a; Palou et al. 2011), this restriction might be associated with a reduction in milk production and hence does not seem to be feasible to extrapolate to humans, if applicable.

Hence, the present study was conducted to determine whether the potential benefits described in the offspring with 30% maternal calorie restriction during lactation in rats on body weight gain in adulthood could also be achieved with a less severe calorie restriction (20%), which could be more easily representative or applicable in humans. If so, the main objective of this study was to use this model to identify early adaptations occurring at the transcriptional level in key tissues involved in energy homeostasis, such as retroperitoneal white adipose tissue (rWAT) and liver, which may account for the protection against obesity development and improved metabolic health found in their offspring. In a second step, we also aimed to assess whether these changes in gene expression levels at early stages were maintained in adulthood, evaluating their behavior under stressful dietary conditions, such as HF-diet feeding, and their potential relation with the improvement of metabolic health.

Methods and Materials

Animals and Experimental Design

The animal protocol followed in this study was reviewed and approved by the Bioethical Committee of the University of the Balearic Islands and guidelines for the use and care of laboratory animals of the University were followed.

The study was conducted on male and female Wistar rats from 16 different litters following the protocol as is described below. Animals were housed under standard conditions, that is, controlled temperature (22 ºC), a 12h light-dark cycle, and free access to tap water. Briefly, 16 virgin female Wistar rats weighing between 225g and 260g were mated with male rats (Charles River Laboratories, Barcelona, Spain). After matching, each female was placed in an individual cage. At day 1 after delivery, pups were weighed and the size of all litters was adjusted to 10 neonates per mother (five males and five females, when possible) to ensure adequate and standardized nutrition until weaning. Dams were assigned to either control (n= 11 dams) or calorie restricted (n= 5 dams) group. Throughout the lactation period, starting on day 1 after delivery until weaning (day 21), control dams were fed *ad libitum* with standard

chow diet (3 kcal/g, with 8% calories from fat; Panlab, Barcelona, Spain), while calorie restricted dams were fed daily with 20% calorie restricted diet. Calorie restriction was performed by offering each dam a daily amount of food corresponding to 80% of the calories that they should eat according to their body weight. This amount was calculated considering the calories consumed daily by their control animals under *ad libitum* feeding conditions. During the lactating period, body weight of male and female offspring of control and calorie restricted dams (control and CRL, respectively) was followed.

At the age of 21 days, a set of animals (n= 6 per group) were killed under *ad libitum* feeding conditions by decapitation during the first 2 h at the beginning of the light cycle. WAT depots (gonadal, inguinal, mesenteric and retroperitoneal; gWAT, iWAT, mWAT and rWAT, respectively) and liver were rapidly removed, weighed, frozen in liquid nitrogen and stored at -80ºC until RNA analysis. Trunk blood samples were also collected in heparinized containers. Plasma was obtained by centrifugation of heparinized blood at 1000 x g for 10 min and stored at -20ºC until analysis of circulating parameters.

Another set of animals (n= 11-16 per group) were weaned at 21 days of life, and were then housed 2 per cage (paired with another animal from the same group), and fed on a normal-fat (NF) standard chow diet (3.8 kcal/g, with 10% calories from fat; Research Diets, NJ, ScholarOne, 375 Greenbrier Drive, Charlottesville, VA, 22901 USA) until the age of 4 months. Then, male and female rats from both control and CRL groups were distributed into two groups: one group (n= 6-8 animals per group) that continued with NF-diet, and another group (n= 5-8 animals per group) that was exposed to HF-diet (4.7 kcal/g, with 45% calories from fat; Research Diets, Inc., NJ, USA) until the age of 6 months. HF-diet contained 5.5% calories from soybean oil and 39.5% from lard. Body weight and food intake of the offspring were recorded from weaning until the age of 6 months, when animals were killed. Body length (from the tip of the nose to the anus) and body composition (by EchoMRI-700TM, Echo Medical Systems, LLC., TX, USA) were measured without anesthesia when animals were 21 days old and 6 months old.

Two weeks before sacrifice, blood samples of control and CRL animals were collected under *ad libitum* feeding conditions and after 12 h fasting. Plasma was obtained as described above to analyze circulating parameters. Finally, at the age of 6 months, NF- and HF-diet fed rats were killed under *ad libitum* feeding conditions by decapitation during the first 2 h at the beginning of the light cycle and on different consecutive days (including animals from each group every day). At killing, different WAT depots (gWAT, iWAT, mWAT and rWAT) and

liver were rapidly removed, weighed, frozen in liquid nitrogen and stored at -80ºC until RNA analysis.

Measurement of circulating parameters and hepatic lipid content

Blood glucose concentration was measured by Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Commercial rat enzyme-linked immunosorbent assay (ELISA) kits were used for the quantification of circulating plasma levels of hormones: insulin (Mercodia AB, Uppsala, Sweden), and adiponectin and leptin (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Commercial enzymatic colorimetric kits were used for the determination of plasma triglyceride (TG) levels (Triglyceride (INT) 20, Sigma Diagnostics, St Louis, MO, USA) and non-esterified fatty acid (NEFA) (Wako Chemicals GmbH, Neuss, Germany). Absorbance of the samples was read with a spectrophotometer Tecan Sunrise Absorbance Reader.

The homeostatic model assessment for insulin resistance (HOMA-IR) was used to assess insulin resistance. It is calculated from fasting insulin and glucose concentration using the formula of Matthews *et al.* (Matthews et al. 1985): HOMA-IR= fasting glucose (mmol/L) x fasting insulin (mU/L)/22.5.

Total lipids in the liver were extracted and quantified by the procedure of Folch *et al.* (FOLCH et al. 1957).

Oral Fat Tolerance Test (OFTT)

At the age of 4 months, an OFTT was performed on control and CRL animals. Animals were deprived of food for 16 h and a load of 2.5 mL/kg body weight of virgin olive oil was orally given to the rats by oral gavage. Blood samples were taken from the saphenous vein, without anesthesia, into heparinized containers before oil load (at time zero), and at 1.5, 3, 4, 6 and 8 h thereafter. Plasma triglyceride levels were measured as described previously.

Gene expression analysis in 21-day and 6-months old offspring

RNA extraction

Total RNA was extracted from liver and rWAT of control and CRL animals by EZNA® TOTAL RNA kit I (Omega Bio-Tek Inc., Norcross, GA, USA) following the manufacturer's

instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Techonologies, Ins., Wilmington, DE) and its integrity confirmed using agarose 1% gel electrophoresis.

Real-time quantitative PCR-analysis

Real-time quantitative polymerase chain reaction (RT-qPCR) was used to measure mRNA expression levels of Cpt1a (carnitine palmitoyltransferase 1 isoform a) and Srebp1c (sterol regulatory element-binding protein 1c) in liver; Cpt1b (carnitine palmitoyltransferase 1 isoform b), and Pparγ (peroxisome proliferator activated receptor gamma 2) in rWAT; and Atgl (adipose triglyceride lipase), Fasn (fatty acid synthase), Gpat (glycerol-3-phosphate acyltransferase), Insr (insulin receptor), Irs1 (insulin receptor substrate 1), Pparα (peroxisome proliferator activated receptor alpha) and Obrb (long form leptin receptor) in liver and rWAT. Rho GDP dissociation inhibitor alpha (Gdi) was used as a housekeeping gene. All primers used for RT-qPCR amplification were obtained from Sigma Genosys (Sigma Aldrich Química SA, Madrid, Spain) and sequences are described in (Palou et al. 2012; Priego et al. 2008; Palou et al. 2009) except for Irs1, whose sequences were: forward 5'-GCAACCGCAAAGGAAATG-3' and reverse 5'-ACCACCGCTCTCAACAGG-3'.

Total RNA (0.25 µg; in a final volume of 5 µl) was denatured at 65ºC for 10 min and then reverse transcribed to cDNA using murine leukemia virus reverse transcriptase (Applied Biosystem, Madrid Spain) at 20ºC for 15 min, 42ºC for 30 min, with a final step of 5 min at 95ºC in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem). Each qPCR was performed from diluted (1/5 or 1/10) cDNA template, forward and reverse primers (5 µM and 10 µM), and Power SYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA). qPCR was performed using the Applied Biosystems Step OnePlusTM Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 min at 95ºC, followed by a total of 40 two temperature cycles (15 s at 95ºC and 1 min at 60ºC). To verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle was calculated by the instrument's software (StepOne Software version 2.2). Relative gene expression numbers were calculated as a percentage of male control rats, using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001).

Statistical analysis

Data are mean ± standard error of the mean (S.E.M.). Multiple comparisons were assessed by ANOVA repeated measures and two-way ANOVA to determine the effects of different factors (calorie restriction during lactation, sex, and feeding conditions) separately for each diet (NF and HF). Single comparisons between groups were assessed by Student's *t* test and Paired *t* test. A correlation analysis was performed for all the genes analyzed with hepatic lipids and circulating parameters considering all the animals as a whole at the age of 6 months. Only the genes showing significant correlations were indicated and were given their Pearson's correlation index (r). To perform the correlation analysis, data of gene expression of all animals were referred to the expression levels of control males under NF-diet. Threshold of significance was set at $p<0.05$, unless indicated. Analysis was performed with SPSS for Windows (SPSS version 19.0, Chicago, IL).

Results

Phenotypic traits and food intake

20% calorie restriction during lactation resulted in lower body weight in the offspring from postnatal day 5 onwards, compared with their controls (Figure 1). This lower body weight was persistent in adulthood, under NF- and HF-diet conditions, and was more pronounced in females than in males. When animals were 6 months old, CRL females weighed 11.4% and 15.7% less than controls, under NF- and HF-diet respectively (Table 1). Notably, body weight of CRL females exposed to HF-diet was even numerically lower than that of CRL females under NF-diet, although differences were not significant. CRL males weighed 7.8% and 6.4% less than their controls, under NF- and HF-diet, respectively.

The lower body weight of CRL animals could be due to shorter body length, for this reason we measured the length from the tip of the nose to the anus (body length) at different ages (Table 1). At weaning (21 days), both male and female CRL pups showed a significant shorter body length than their controls ($p<0.05$, two-way ANOVA), but no significant differences were found in adulthood in either sex.

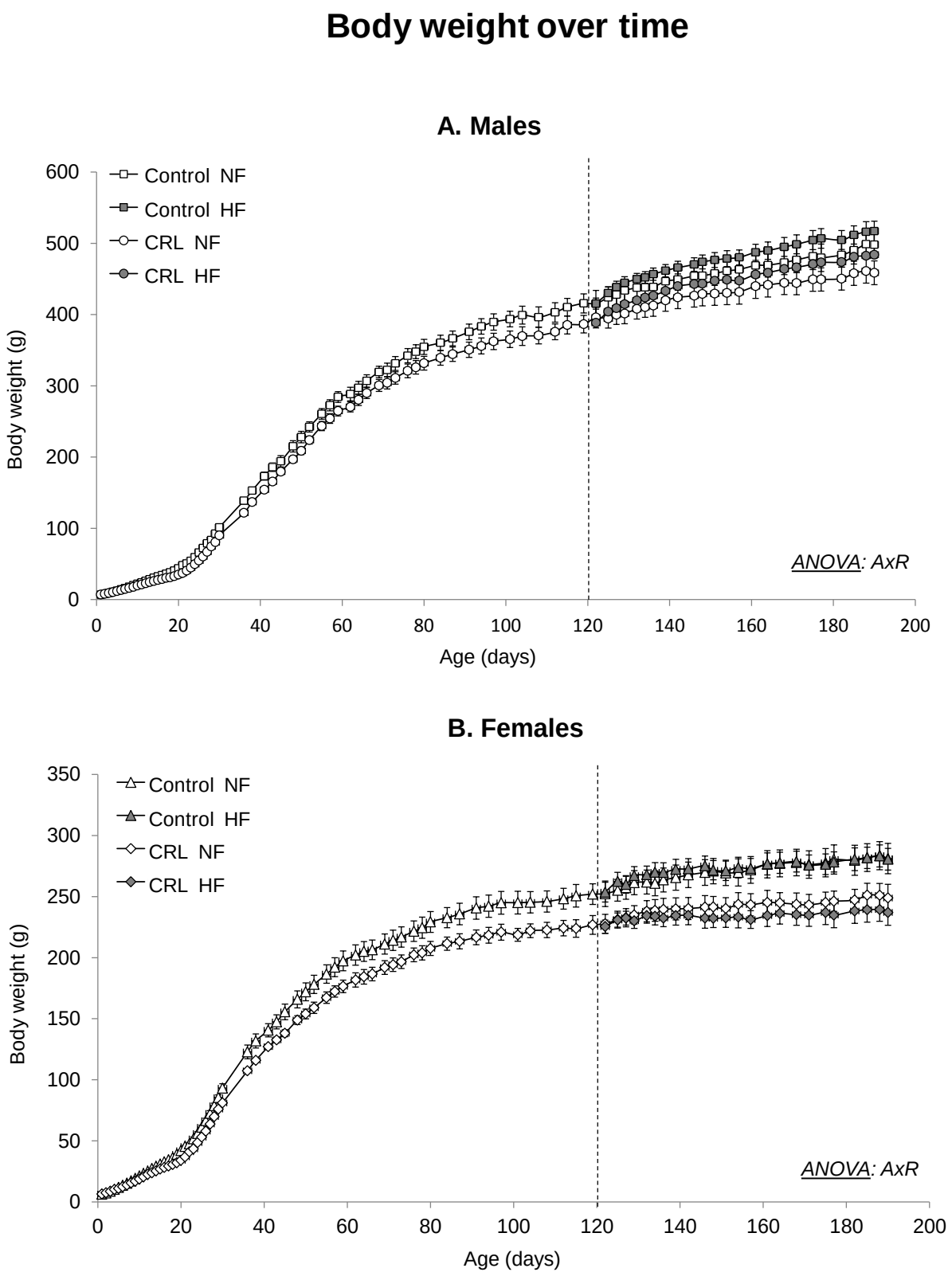

Figure 1. Body weight over time of male and female offspring of control and calorie restricted dams during lactation (CRL) from day 1 until day 190 of age. From the age of 120 days, half of the animals of both control and CRL group were exposed to a HF-diet, and the other half remained with NF-diet. The dashed line shows the beginning of the HF-diet. Data are mean ± S.E.M. (n= 11-16 animals per group until the age of 120 days; n= 5-8 animals per group until the age of 190 days). Statistics: *AxR*, interaction between age and maternal calorie restriction during lactation ($p<0.05$, ANOVA repeated measures).

Table 1. Phenotypic traits at 21 days old and 6 months old, and cumulative food intake before and after exposure to HF-diet.

21 days old	**Males**		**Females**		*ANOVA*
	Control	**CRL**	**Control**	**CRL**	
Body weight (g)	48.1± 2.1	37.1 ± 0.8*	45.4 ± 1.1	36.9 ± 0.5*	*R*
Body length (cm)	11.5 ± 0.2	10.8 ± 0.2*	11.0 ± 0.2	10.4 ± 0.1*	*R,S*
Body fat mass (%)	10.0 ± 0.4	8.68 ± 0.26*	10.5 ± 0.3	8.87 ± 0.19*	*R*
Body lean mass (%)	83.8 ± 1.0	87.2 ± 0.7*	83.4 ± 0.6	86.9 ± 1.0*	*R*
gWAT (mg)	65.2 ± 8.3	47.8 ± 4.6	38.8 ± 5.2	23.2 ± 2.4*	*R*
iWAT (mg)	294 ± 35	187 ± 19*	307 ± 33	186 ± 26*	*R*
mWAT (mg)	105 ± 4	81.8 ± 2.2*	98.9 ± 11.2	81.2 ± 6.1	*R*
rWAT (mg)	69.6 ± 9.9	40.1 ± 4.9*	50.5 ± 3.7	24.9 ± 3.2*	*R,S*
6 months old – NF diet	**Males**		**Females**		*ANOVA*
	Control	**CRL**	**Control**	**CRL**	
Body weight (g)	498 ± 19	459 ± 17	281 ± 12	249 ± 11	*R,S*
Body length (cm)	24.2 ± 0.5	24.0 ± 0.4	20.9 ± 0.4	20.3 ± 0.2	*S*
Body fat mass (%)	20.7 ± 2.2	22.1 ± 1.6	22.1 ± 2.3	14.5 ± 1.2	*RxS*
Body lean mass (%)	68.8 ± 2.2	67.6 ± 1.6	67.0 ± 2.1	73.9 ± 0.9*	*RxS*
gWAT (g)	16.7 ± 2.0	12.1 ± 0.6*	9.96 ± 1.42	5.40 ± 0.90*	*R,S*
iWAT (g)	9.95 ± 1.20	10.7 ± 0.7	3.93 ± 0.37	2.14 ± 0.45*	*RxS*
mWAT (g)	7.09 ± 0.51	8.06 ± 0.79	3.60 ± 0.40	2.02 ± 0.34*	*RxS*
rWAT (g)	12.8 ± 1.7	12.4 ± 1.0	4.53 ± 0.67	2.16 ± 0.23*	*RxS*
6 months old – HF diet	**Males**		**Females**		*ANOVA*
	Control	**CRL**	**Control**	**CRL**	
Body weight (g)	517 ± 14	484 ±34	281 ± 9	237 ± 10	*R,S*
Body length (cm)	24.3 ± 0.3	24.1 ± 0.4	20.6 ± 0.3	20.3 ± 0.3	*S*
Body fat mass (%)	30.5 ± 1.6	27.7 ± 2.4	23.0 ± 1.1	19.3 ± 1.4*	*R(p=0.067), S*
Body lean mass (%)	56.9 ± 2.3	63.9 ± 1.0*	66.3 ± 1.1	69.2 ± 1.5	*R,S*
gWAT (g)	23.0 ± 1.7	18.9 ± 2.7	11.4 ± 1.1	7.18 ± 1.33*	*R,S*
iWAT (g)	15.4 ± 1.4	13.8 ± 2.3	4.27 ± 0.53	2.93 ± 0.41	*R (p=0.078),S*
mWAT (g)	10.2 ± 1.8	10.9 ± 1.6	3.10 ± 0.26	2.27 ± 0.39	*S*
rWAT (g)	21.2 ± 2.0	18.9 ± 3.2	5.04 ± 0.47	3.13 ± 0.43*	*R,S*
Cumulative food intake (Kcal)	**Males**		**Females**		*ANOVA*
	Control	**CRL**	**Control**	**CRL**	
From day 21 to 120 under NF-diet	6531 ± 102	6371 ± 85	4935 ± 65	4630 ± 101*	*R,S*
From day 121 to 190 under NF-diet	4701 ± 152	4419 ± 71	3062 ± 67	2776 ± 106*	*R,S*
From day 121 to 190 under HF-diet	5118 ± 123	4902 ± 55	3298 ± 74	2716 ± 88*	*R,S*

Abbreviations: gonadal white adipose tissue (gWAT), inguinal white adipose tissue (iWAT), mesenteric white adipose tissue (mWAT), retroperitoneal white adipose tissue (rWAT), normal fat diet (NF-diet) and high fat diet (HF-diet). Data are mean ± S.E.M (n=5-8 animals per group) of male and female offspring of control and calorie restricted dams during lactation (CRL). Statistics: R, effect of maternal calorie restriction during lactation; S, effect of sex; RxS, interaction between maternal calorie restriction during lactation and sex ($p<0.05$, two-way ANOVA). *, CRL vs. Control ($p<0.05$, Student's t test).

At weaning, CRL animals showed higher body lean content and lower body fat content than controls; they also showed a decrease in weight of the main (gonadal, inguinal, mesenteric and retroperitoneal) WAT depots ($p<0.05$, two-way ANOVA) (Table 1). At the age of 6 months, differences concerning body fat content between control and CRL animals were found under HF-diet ($p= 0.067$, two-way ANOVA), particularly in females ($p<0.05$, Student's *t* test). A similar trend was found when analyzing the weight of different WAT depots. Concerning body lean content, HF-diet fed CRL animals showed significantly higher lean content than controls ($p<0.05$, two-way ANOVA). Under NF-diet, only CRL females,

but not males, showed a higher percentage of lean mass and a tendency to lower fat content (interactive effect between sex and calorie restriction, $p<0.05$, two-way ANOVA). The same tendency was seen for the weight of main WAT depots, with the exception of the gonadal one, which showed a significant decrease in both male and female CRL animals ($p<0.05$, two-way ANOVA).

Food intake was also measured to determine whether differences in body weight gain between control and CRL animals could be explained by differences in food intake (Table 1). CRL animals ate fewer calories than controls, both when exposed to NF- or HF-diet conditions ($p<0.05$, two-way ANOVA), with the differences being more marked and significant by Student's *t* test in females.

Oral fat tolerance test

At 4 months of age, we measured TG response to an OFTT in male and female offspring of control and calorie restricted dams (Figure 2). CRL male animals showed a tendency to lower area under the curve (AUC) from 0-8h (7.9 ± 0.6 mg·h /mL) compared with their controls (10.4 ± 1.1 mg·h /mL) ($p= 0.071$, Student's *t* test). These animals also showed a tendency to lower TG levels at the time point of 4 h ($p= 0.072$, Student's *t* test). No significant changes were found between control and CRL females.

Circulating parameters

Circulating glucose, insulin, leptin, adiponectin, NEFA, and TG levels of control and CRL animals at the age of 21 days and 6 months are shown in Table 2. At weaning, compared to controls, male and female CRL pups displayed lower circulating levels of glucose, insulin and leptin ($p<0.05$, two-way ANOVA) and higher adiponectin levels (only CRL males, $p<0.05$ Student's *t* test). CRL pups also showed lower leptin-to-adiponectin (L/A) ratio than controls ($p<0.05$, Student's *t* test). No differences were found concerning NEFA and TG levels between control and CRL pups.

At the age of 6 months, under NF-diet conditions, no significant differences were found in glucose levels between control and CRL rats, although fasted CRL females showed lower glucose levels than controls ($p<0.05$, Student's *t* test). Notably, only CRL animals showed a significant decrease in glucose levels as an effect of fasting conditions ($p<0.05$, Paired *t* test). CRL animals, and particularly females, showed lower levels of insulin than their controls

(p<0.05, ANOVA repeated measures), and CRL females also showed lower leptin and L/A ratio (p<0.05, Student's *t* test; interactive effect between calorie restriction and sex, p<0.05, two-way ANOVA).

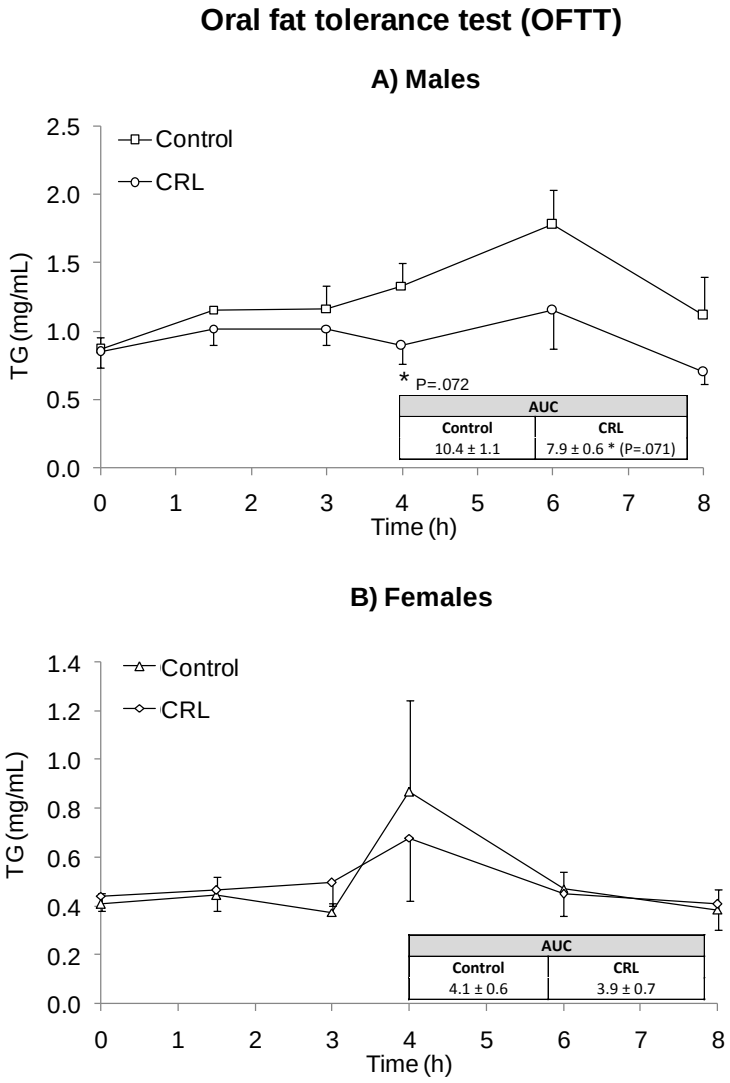

Figure 2. Triglyceride (TG) response (mg/mL) to an oral fat tolerance test (OFTT) in male (A) and female (B) offspring of control and calorie restricted dams during lactation (CRL) at 4 months of age. Data are mean ± S.E.M. (n= 6 animals per group). Statistics: *, CRL *vs* Control (p<0.05, Student's *t* test). Abbreviations: area under the curve (AUC).

We did not find significant differences between control and CRL animals concerning HOMA index under NF-diet, but female CRL animals showed a tendency to lower values than controls (p= 0.076, Student's *t* test). No significant differences were found in adiponectin, NEFA or TG levels due to maternal calorie restriction in NF-diet fed rats. When exposed to HF-diet, a sex- and treatment- dependent response to feeding conditions was found in glucose levels (interactive effect between sex, calorie restriction and feeding conditions, p<0.05, ANOVA repeated measures); this is explained by the lower fasted glucose levels of CRL females, but not males, with respect to their controls (p<0.05, Student's *t* test) and with respect to the fed state (p<0.05, Paired *t* test). Interestingly, CRL animals showed lower

leptin, L/A ratio and TG levels than control rats ($p<0.05$, two-way ANOVA). No significant differences were found in insulin, adiponectin or NEFA levels as an effect of calorie restriction during lactation under a HF-diet. However, HF-diet exposed CRL females, but not males, displayed significantly lower HOMA index than their controls ($p<0.05$, Student's *t* test).

Table 2. Circulating parameters under *ad libitum* feeding conditions and after 12h fasting conditions (for glucose and insulin plasma levels) at the age of 21 days under NF-diet, and 6 months under NF- and HF-diet.

21 days old		**Males**		**Females**		*ANOVA*
		Control	**CRL**	**Control**	**CRL**	
Glucose (mg/dL)	Fed	137 ± 7	120± 3*	141± 5	116± 4*	*R*
Insulin (µg/L)	Fed	0.111 ± 0.042	0.044 ± 0.010	0.105 ± 0.020	0.037 ± 0.004*	*R*
Leptin (µg/L)	Fed	0.941 ± 0.154	0.408 ± 0.045*	1.05 ± 0.11	0.585 ± 0.162*	*R*
Adiponectin (µg/mL)	Fed	4.24 ± 0.47	9.15 ± 1.54*	9.72 ± 0.64	10.6 ± 1.3	*RxS*
L/A ratio (%)	Fed	100 ± 13	32.1 ± 9.7*	58.4 ± 3.9	31.3± 7.4*	*RxS*
NEFA (mM)	Fed	1.46 ± 0.13	1.50 ± 0.14	1.37 ± 0.19	1.59 ± 0.13	
TG (mg/mL)	Fed	1.02 ± 0.14	0.893 ± 0.130	0.938 ± 0.094	0.851 ± 0.042	
6 months old – NF diet		**Males**		**Females**		*ANOVA*
		Control	**CRL**	**Control**	**CRL**	
Glucose (mg/dL)	Fed	108 ± 3	107 ± 6	105 ±4	102 ± 4	*F*
	Fasting	98 ± 8	95 ± 3$^{\Omega}$	102 ± 3	90 ± 1$^{*,\Omega}$	
Insulin (µg/L)	Fed	2.77 ± 0.94	1.44 ± 0.26	0.835 ± 0.102	0.459 ± 0.050*	*FxR,S*
	Fasting	0.920 ± 0.379 $^{\Omega}$	1.06 ± 0.24 $^{\Omega}$	0.558 ± 0.121 $^{\Omega}$	0.287 ± 0.047 $^{\Omega}$	
HOMA-IR	Fasting	5.37 ± 2.38	5.77 ± 1.01	3.33 ± 0.75	1.67 ± 0.32	*S*
Leptin (µg/L)	Fed	15.8 ± 2.2	13.4 ± 1.1	6.07 ± 0.94	1.74 ± 0.19*	*RxS*
Adiponectin (µg/mL)	Fed	6.73 ± 0.60	6.60 ± 0.62	7.79 ± 0.63	8.05 ± 0.57	*S*
L/A ratio (%)	Fed	100 ± 12	90.8 ± 9.9	35.0 ± 5.6	9.39 ± 1.16*	*RxS*
NEFA (mM)	Fed	0.851 ± 0.152	0.910 ± 0.056	1.33 ± 0.24	0.866 ± 0.086	
TG (mg/mL)	Fed	1.61 ± 0.08	1.40 ± 0.19	1.02 ± 0.12	0.920 ± 0.073	*S*
6 months old – HF diet		**Males**		**Females**		*ANOVA*
		Control	**CRL**	**Control**	**CRL**	
Glucose (mg/dL)	Fed	117 ± 4	108 ± 2	106 ± 4	106 ± 4	*FxRxS*
	Fasting	103 ± 6	99.7 ± 4.0	106 ± 3	83 ± 1$^{*,\Omega}$	
Insulin (µg/L)	Fed	1.57 ± 0.33	1.79 ± 0.50	0.550 ± 0.093	0.341 ± 0.067	*F,S*
	Fasting	1.05 ± 0.21	1.16 ± 0.34	0.424 ± 0.079	0.254 ± 0.047	
HOMA-IR	Fasting	6.81± 1.05	5.41± 1.08	2.69± 0.50	1.28± 0.23*	*R (p=0.063),S*
Leptin (µg/L)	Fed	20 ± 3.6	16.5 ± 3.2	5.79 ± 0.84	2.73 ± 0.30*	*R,S*
Adiponectin (µg/mL)	Fed	6.86 ± 0.47	7.45 ± 0.36	7.58 ± 0.54	8.07 ± 0.53	*S*
L/A ratio (%)	Fed	100 ± 16	77.1 ± 15.5	25.9 ± 3.1	11.5 ± 1.7*	*R,S*
NEFA (mM)	Fed	0.973 ± 0.120	1.15 ± 0.13	0.917 ± 0.059	0.825 ± 0.088	
TG (mg/mL)	Fed	1.35 ± 0.14	0.962 ± 0.046*	0.662 ± 0.144	0.480 ± 0.094	*R,S*

Abbreviations: insulin resistance HOMA index (HOMA-IR),leptin-to-adiponectin ratio (L/A ratio), non-esterified fatty acid (NEFA), triglycerides (TG).Data are mean ± S.E.M (n=5-8 animals per group) of male and female offspring of control and calorie restricted dams during lactation (CRL). Statistics:*F*, effect of feeding conditions;*R*, effect of maternal calorie restriction during lactation; *S*, effect of sex;*FxR*, interaction between feeding conditions and maternal calorie restriction during lactation*; RxS*, interaction between maternal calorie restriction during lactation and sex; *FxRxS*, interaction between feeding conditions, maternal calorie restriction during lactation and sex($p<0.05$, two-way ANOVA). *, CRL *vs* Control; $^{\Omega}$, fasting*vs*feeding conditions($p<0.05$, Student's *t* test).

Hepatic lipid content

Figure 3 shows hepatic lipid content in the different groups of animals at the age of 6 months. CRL animals presented lower lipid content compared to controls, both under NF- and HF-diet conditions ($p<0.05$, Student's *t* test). Interestingly, hepatic lipid content increased in male animals when exposed to HF-diet, but this increase was less marked in CRL animals ($p<0.05$, Student's *t* test). In contrast, HF-diet feeding, in the conditions of this study, did not induce significant changes in hepatic lipid content in females, either in control or CRL animals.

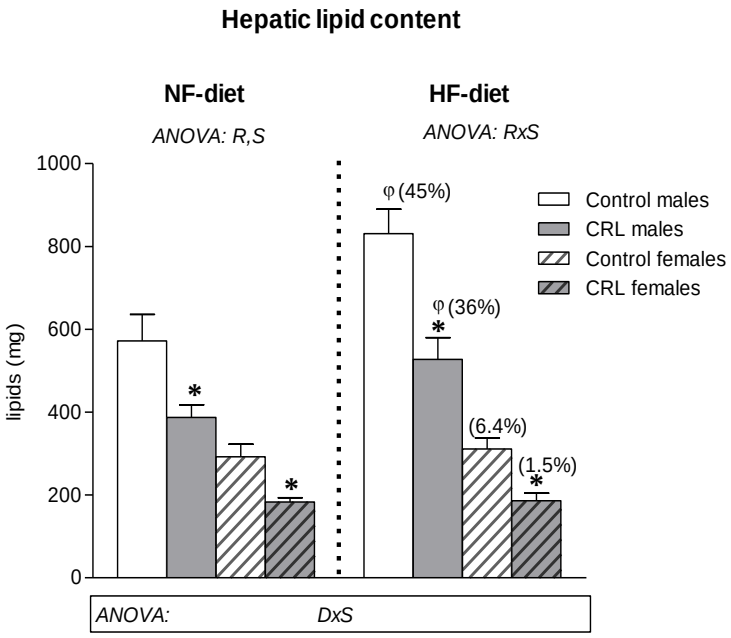

Figure 3. Hepatic lipid content (mg) in male and female offspring control and calorie restricted dams during lacation (CRL) at 6 months of age under NF- and HF-diet. The number in brackets indicates the percentage of lipid increment in liver as effect of HF-diet *vs* their respective controls under NF-diet. Data are mean ± S.E.M. (n= 6-8 animals per group under NF-diet; n= 5-8 animals per group under HF-diet). Statistics: *R*, effect of maternal calorie restriction during lactation; *S*, effect of sex; and *RxS*, interaction between maternal calorie restriction during lactation and sex; *DxS*, interaction between diet and sex ($p<0.05$, two-way ANOVA). *, CRL *vs* Controls; φ, HF-diet *vs* NF-diet ($p<0.05$, Student's *t* test). Abbreviations: Normal-fat diet (NF-diet), high-fat diet (HF-diet).

mRNA levels of energy balance and lipid metabolism related-genes in rWAT and liver

Figure 4A shows mRNA expression levels of selected genes in rWAT of 21-day-old control and CRL pups. The retroperitoneal depot was selected to be analyzed for gene expression, based on the literature showing that this depot seems to be more sensitive to nutritional status, compared with other depots (Palou et al. 2010b). CRL pups showed lower mRNA levels of Fasn and greater mRNA levels of Pparα, Atgl, Insr and Irs1 than controls ($p<0.05$, two-way ANOVA). In addition, an interaction between the effects of calorie restriction during lactation and sex was observed in the mRNA levels of Gpat ($p<0.05$, two-way ANOVA), since CRL female animals, but not males, showed a significant decrease in their expression levels ($p<0.05$, Student's *t* test). No significant differences were observed in the expression levels of the other genes analyzed (Pparγ, Cpt1b, and Obrb) between control and CRL

animals. Figures 4B and 4C show mRNA levels of 6-month-old controls and CRL animals in rWAT under NF- and HF-diet conditions, respectively. Changes described in 21-day-old pups between control and CRL animals were not maintained in adulthood under NF-diet conditions; adult CRL animals showed even higher mRNA levels of Gpat than controls ($p<0.05$, two-way ANOVA), with the difference being more marked and significant in males ($p<0.05$, Student's *t* test). However, under HF-diet, CRL rats displayed higher mRNA levels of Atgl and Irs1 compared to their controls ($p<0.05$, two-way ANOVA), similarly to what was found at the age of 21 days. In addition, CRL animals showed higher mRNA levels of Obrb and, contrary to what was found in young animals, higher Fasn mRNA levels than controls ($p<0.05$, two-way ANOVA). Moreover, CRL females showed increased Cpt1b mRNA expression with respect to controls ($p<0.05$, Student's *t* test), while no significant changes were found in CRL males (interactive effect between sex and calorie restriction, $p<0.05$, two-way ANOVA).

Results on gene expression in liver of 21-day-old control and CRL pups are shown in Figure 5A. CRL animals showed lower mRNA levels of Srebp1c and higher mRNA levels of Cpt1a and Obrb compared to their controls ($p<0.05$, two-way ANOVA). CRL animals also showed Atgl mRNA levels slightly higher than controls ($p= 0.087$, two-way ANOVA). No significant differences were found concerning mRNA levels of Fasn, Gpat, Pparα, Insr or Irs1 as an effect of calorie restriction during lactation.

Figures 5B and 5C show mRNA expression levels in the liver of 6-month-old control and CRL animals under NF- and under HF-diet, respectively. NF-diet fed CRL animals showed lower mRNA levels of Gpat ($p<0.05$, two-way ANOVA) and Srebp1c ($p=0.072$, two-way ANOVA, especially CRL males by Student's *t* test, $p<0.05$) with respect to controls. No significant differences between control and CRL animals were found for the rest of genes studied under NF-diet conditions, although an interaction between maternal calorie restriction during lactation and sex was found for mRNA levels of Cpt1a. When exposed to HF-diet, sex-dependent differences between control and CRL animals were found. Concretely, no changes or a trend to lower mRNA levels of Srebp1c, Pparα, Insr, Irs1 and Obrb were found in CRL males with respect to their controls, whereas higher mRNA levels (Irs1 and Obrb) or a tendency to higher levels (Srebp1c, Pparα, Insr) was found in females (interactive effect between calorie restriction and sex, $p<0.05$, two-way ANOVA).

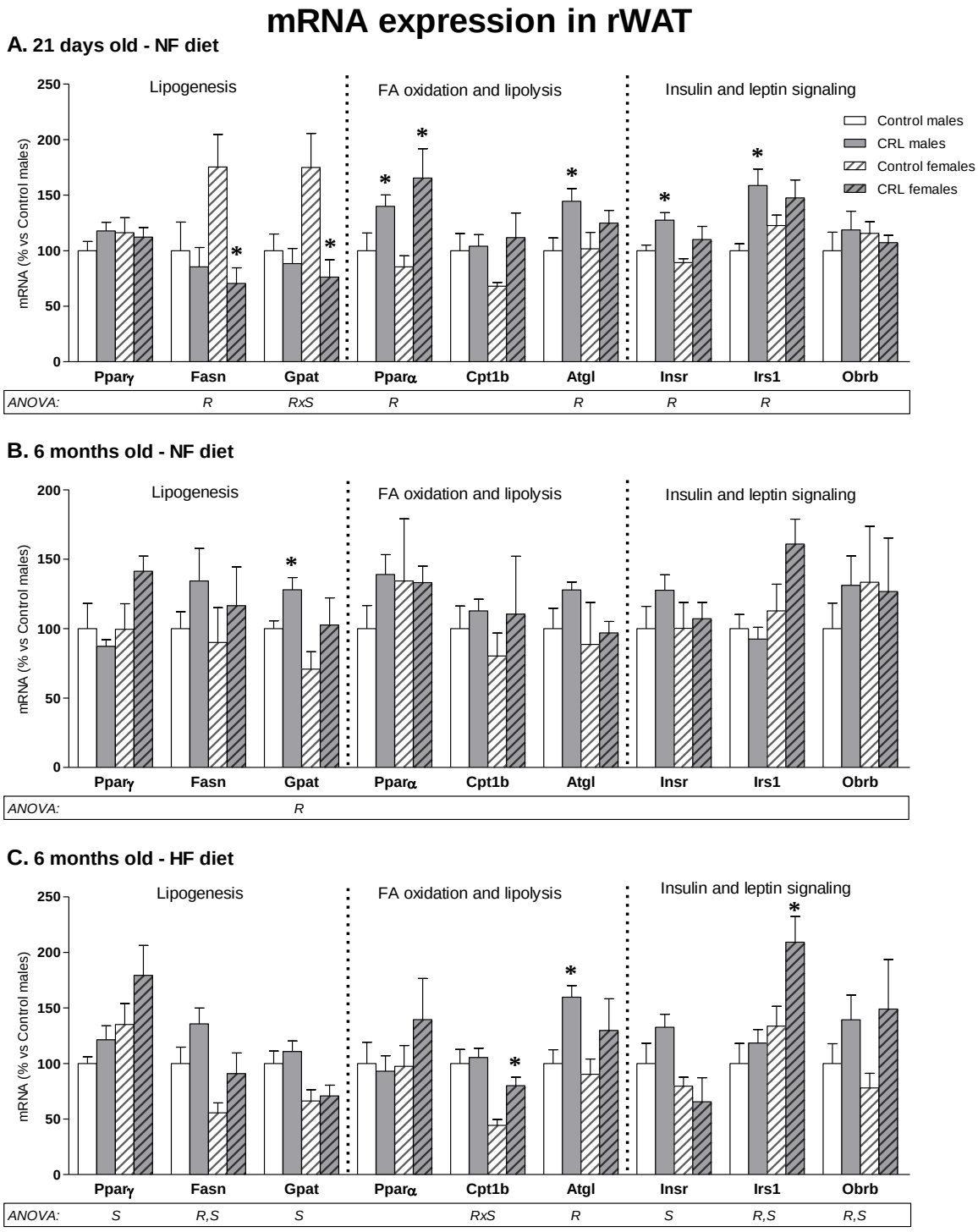

Figure 4. mRNA levels of energy balance and lipid metabolism related genes in retroperitoneal white adipose tissue (rWAT) of male and female offspring of control and calorie restricted dams during lactation (CRL), at 21 days old (A), 6 months old under NF-diet (B), and 6 months old under HF-diet (C). mRNA levels were measured by RT-qPCR and expressed as a percentage of the mean value of control males. Data are mean ± S.E.M. (n= 6-7 animals per group at 21 days; n= 6-8 animals per group at 6 months under NF-diet; n= 5-8 animals per group at 6 months under HF-diet). Statistics: R, effect of maternal calorie restriction during lactation; S, effect of sex; and RxS, interaction between maternal calorie restriction during lactation and sex ($p<0.05$, two-way ANOVA). *, CRL vs Controls ($p<0.05$, Student's t test). Abbreviations: peroxisome proliferator activated receptor gamma (Pparγ), fatty acid synthase (Fasn), glycerol-3-phosphate acyltransferase (Gpat), peroxisome proliferator activated receptor alpha (Pparα), carnitine palmitoyltransferase 1 isoform b (Cpt1b), adipose triglyceride lipase (Atgl), insulin receptor (Insr), insulin receptor substrate 1 (Irs1) and long form leptin receptor (Obrb).

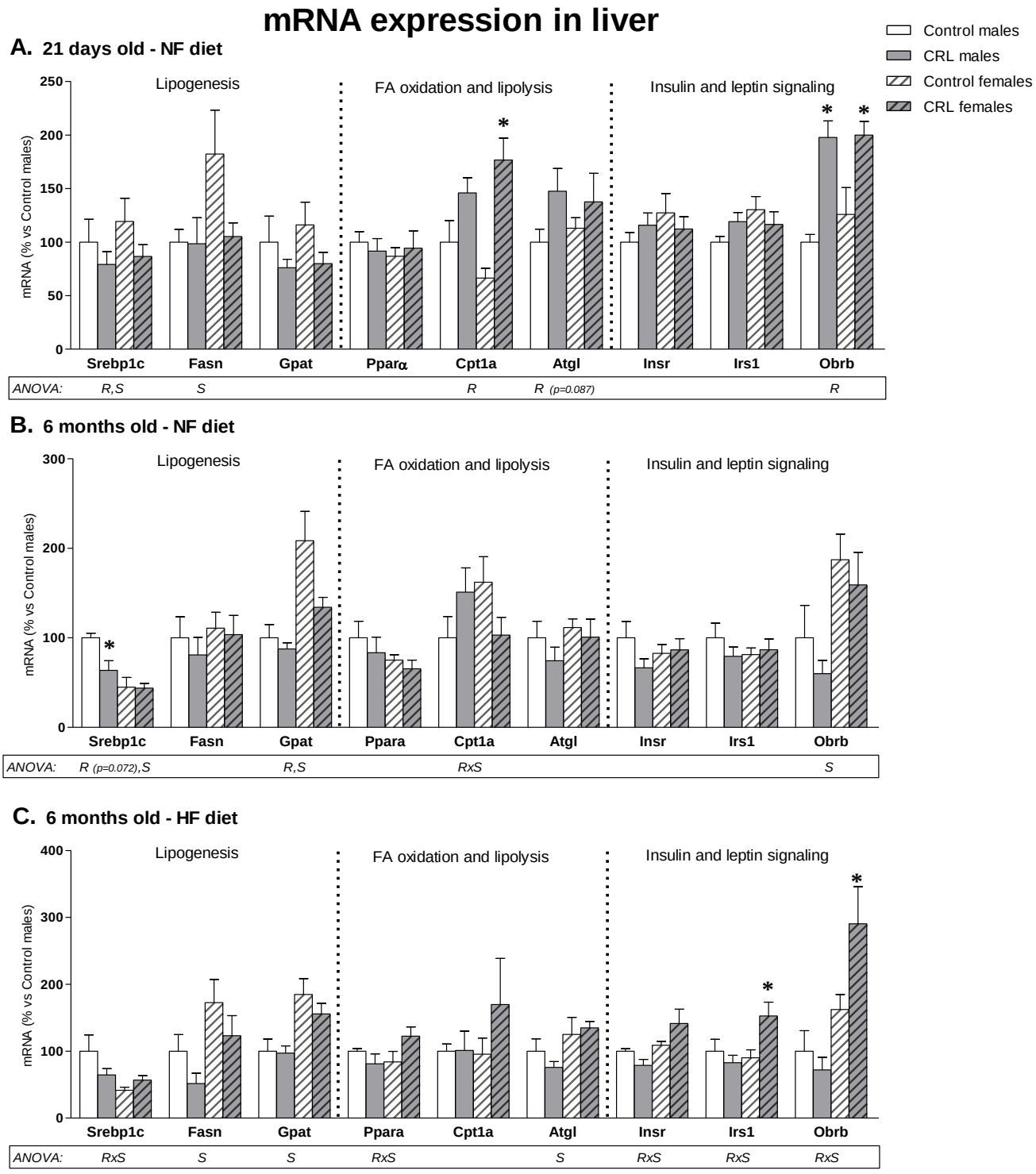

Figure 5. mRNA levels of energy balance and lipid metabolism related genes in liver of male and female offspring of control and calorie restricted dams during lactation (CRL), at 21 days old (A), 6 months old under NF-diet (B), and 6 months old under HF-diet (C). mRNA levels were measured by RT-qPCR and expressed as a percentage of the mean value of control males. Data are mean ± S.E.M. (n= 6-7 animals per group at 21 days; n= 6-8 animals per group at 6 months under NF-diet; n= 5-8 animals per group at 6 months under HF-diet). Statistics: R, effect of maternal calorie restriction during lactation; S, effect of sex; and RxS, interaction between maternal calorie restriction during lactation and sex ($p<0.05$, two-way ANOVA). *, CRL vs Controls ($p<0.05$, Student's t test). Abbreviations: sterol regulatory element-binding protein 1c (Srebp1c), fatty acid synthase (Fasn), glycerol-3-phosphate acyltransferase (Gpat), peroxisome proliferator activated receptor alpha (Pparα), carnitine palmitoyltransferase 1 isoform a (Cpt1a), adipose triglyceride lipase (Atgl), insulin receptor (Insr), insulin receptor substrate 1 (Irs1) and long form leptin receptor (Obrb).

Correlation analysis of selected parameters

We used correlation analysis to investigate putative relationships between transcript levels of the genes studied with hepatic lipid content and plasma circulating parameters (Table 3). Considering all animals as a whole at the age of 6 months, we observed a negative correlation between mRNA levels of Irs1 in rWAT with hepatic lipids (r= -0.436; p= 0.001, Pearson's correlation) and with TG plasma levels (r= -0.386; p= 0.005, Pearson's correlation). Obrb mRNA levels in liver also showed a negative correlation with hepatic lipid content (r= -0.477; p= 0.000, Pearson's correlation) and with circulating levels of insulin under *ad libitum* feeding conditions (r= -0.246; p= 0.076, Pearson's correlation). Finally, mRNA levels of hepatic Srebp1c showed a positive correlation with hepatic lipids (r= 0.368; p= 0.013, Pearson's correlation) and with HOMA index (r= 0.276; p= 0.070, Pearson's correlation). No significant correlations were found regarding transcript levels of the other genes studied in rWAT or liver (data not shown).

Table 3. Correlation analysis ofhepatic lipid content and plasma circulating parameters with mRNA expression levelsof Irs1 in rWATand of Obrb and Srebp1c in liver, considering all animals as a whole at the age of 6 months.

		Hepatic Lipids	Plasma TG	Insulin AL	HOMA-IR
Irs1 (rWAT)	r	-0.436	-0.386	---	---
	p	0.001**	0.005**		
Obrb (Liver)	r	-0.477	---	-0.246	---
	p	0.000**		0.076	
Srebp1c (Liver)	r	0.368	---	---	0.276
	p	0.013*			0.070

Correlations were performed for all genes analyzed, but only those showing significant correlations are indicated. r, Pearson correlation index; p, p-values for Pearson correlation coefficient. Abbreviations: ad libitum (AL), insulin resistance HOMA index (HOMA-IR), insulin receptor substrate 1 (Irs1), long form leptin receptor (Obrb), triglycerides (TG), retroperitoneal white adipose tissue (rWAT). Statistics: *, p-value<0.05; **, p-value<0.005.

Discussion

Here, we show that a modest maternal calorie restriction (20%) during lactation, which could be a typical situation of dieting representative or applicable in humans, causes beneficial effects in offspring against obesity development and other metabolic alterations in adult life. Notably, the effects of this condition during lactation improving the offspring metabolic health were generally more evident in females, and particularly when animals were exposed to obesogenic conditions. Therefore, this model can be useful to ascertain what adaptations are taking place at early ages that may confer protection against later development of obesity.

Regarding the characterization of the model, in short, we found that the offspring of 20% calorie restricted dams during lactation showed lower body weight in adulthood, as well as lower adiposity when exposed to HF-diet conditions, with the differences being more evident in females. These effects could be explained, in part, by their lower food intake. Cumulative food intake was lower in CRL animals than their controls, both under NF- and HF-diet, with the differences being also more marked in females. These results are in accordance with our previous studies with 30% calorie restriction, where a comparison of accumulated calories eaten in adulthood also showed a lower value for CRL animals, particularly females, compared to controls (Palou et al. 2010a; Palou et al. 2011). Remmers *et al.* (Remmers et al. 2008a) also observed a reduced body weight, fat content and food intake in calorie restricted rats during lactation obtained by increasing the litter size.

At early ages, the lower body weight and body fat content of CRL animals was associated to shorter body length. However, differences in body length disappeared as the animals grew and no differences were found in adulthood. These results contrast with those described when increasing litter size (Remmers et al. 2008b) which maintained shorter body weight and length than their controls. Other models of nutrient restriction during lactation, such as that obtained by 65% maternal protein restriction also showed more severe effects on body weight in the offspring as well as shorter body length until the age of 6 months (de Moura et al. 2007). Interestingly, the very modest calorie restricted diet used here during the suckling period (20%) also prevents excess weight gain in their offspring and reduces body fat accretion in females, without impeding catching up normal body length under standard diet conditions.

CRL animals also displayed a better profile of circulating parameters. Leptin and insulin are proteins related with energy intake and expenditure and their circulating levels may be

considered as biomarkers of metabolic health and appropriate body weight and food intake control (Friedman and Halaas 1998). Adiponectin is also linked with obesity and insulin sensitivity (Berg et al. 2001; Yamauchi et al. 2001), and the leptin to adiponectin (L/A) ratio has been proposed as a useful, more reliable measure of insulin resistance and vascular risk than levels of leptin and adiponectin alone (Finucane et al. 2009; Satoh et al. 2004). At weaning, plasma levels of glucose, insulin and leptin were significantly lower in CRL animals compared to their controls, remarkably in females. In turn, adiponectin levels were significantly higher in CRL animals, and hence the L/A ratio was significantly lower. Therefore, levels of insulin, leptin and adiponectin in CRL animals at this early age suggest that these animals are programmed for better body weight control. The above differences between control and CRL animals were partially maintained in adulthood, but were more evident under HF-diet conditions. Concretely, under HF-diet, CRL animals showed lower leptin levels and lower L/A ratio than controls; in both cases the improved effects were more marked in females. CRL females also showed lower HOMA index than their controls. Thus, measures of both L/A ratio and HOMA index were indicative of improved insulin sensitivity in CRL animals, particularly females.

Circulating lipid profile could also give some clues to characterize the metabolic health of these animals in relation with lipid metabolism and handling. In this sense, we previously described that 30% moderate maternal calorie restriction in lactating rats resulted in an improved capacity of the adipose tissue of male adult offspring to handle and store excess fuel when exposed to HF-diet, as evidenced by changes at the gene expression level of key genes involved in lipid metabolism (Palou et al. 2011). Thus, to further study whether a more modest 20% calorie restriction during lactation was associated with a better capacity to handle dietary lipids and improved lipemia, an OFTT was performed at the age of 4 months. Impaired postprandial metabolism of TG in terms of a higher peak or delayed clearance has been associated with higher cardiovascular risk (Ansar et al. 2011). Results showed a trend to a lower increase in circulating TG after the oral load of fat in CRL male animals, compared to controls. It must be noted that, although differences between both groups were slight, this improvement in CRL males was already evident at the age of 4 months, and prior to apparent signs of an impaired function related to age. In female animals, differences between control and CRL were not evident, at least at this age. The protective effects of calorie restriction during lactation against dyslipidemia were further evident later on when animals were exposed to HF-diet, since CRL animals, both males and females, showed lower TG levels

than controls under this dietary stressor. Interestingly, CRL animals, both under NF- and HF-diet, also displayed lower hepatic lipid content, the hallmark of nonalcoholic fatty liver disease (NAFLD), which is well recognized as being part of the metabolic syndrome (Postic and Girard 2008). In accordance with the literature (Priego et al. 2008), fat overloading in the diet brings a different response in males and females, since males seems to be more prone to accumulating lipids in the liver. Here it is also shown that, in both sexes, the response to HF-diet seem to be better in CRL animals compared to controls.

All in all, these results, together with our previous studies with a higher restriction (Palou et al. 2010a; Palou et al. 2011), show that moderate calorie restriction during lactation may protect the offspring against the development of obesity and related metabolic alterations in later life, including insulin and leptin resistance, dyslipidemia, and hepatic steatosis. Considering the fact that the incidence of obesity is increasing worldwide at alarming rates, the identification of strategies for its prevention is of great interest, from early stages of life. This model could be useful to ascertain what adaptations during early life could be responsible for later benefits, and hence to identify potential markers of improved metabolic health and body weight control. For this reason we analyzed the expression of selected genes in key tissues involved in energy metabolism, such as rWAT and liver, at a young age (at weaning), to identify early transcript-based markers. Besides, to ascertain the robustness of these potential biomarkers, we also analyzed whether they were persistent in adulthood, under normal or stressful dietary conditions, and if a relationship exists between the levels of these transcripts and circulating parameters.

Regarding rWAT, when looking at the expression of genes related with insulin and leptin sensitivity, results showed that CRL pups, at weaning, displayed higher Insr and Irs1 mRNA expression levels. In adulthood, differences were not maintained under NF-diet; however, under HF-diet conditions, mRNA expression levels of Irs1 were also higher in CRL animals in comparison to controls. Obesity is known to be the most common cause of insulin resistance and to be accompanied by a decrease in insulin receptor density and the related failure to activate tyrosine kinase activity (Brock and Dorman 2007). Thus, the fact that CRL animals showed higher mRNA levels of proteins involved in insulin signaling in rWAT suggests that these animals may be programmed to better respond in this tissue to the biological actions of insulin. Interestingly, mRNA levels of Irs1 in adult animals were negatively correlated with hepatic lipid content and with TG circulating levels. Thus, although correlation does not imply causation, this association pinpoints the potential

relevance of transcript levels of this gene as a marker of metabolic health, programming animals for a better body weight control. Regarding leptin signaling, mRNA expression levels of Obrb at weaning were not changed by the effects of maternal calorie restriction during lactation, nevertheless, in adulthood and under HF-diet, Obrb mRNA levels were increased in CRL animals compared to controls.

In addition, 21-day-old CRL pups showed a better expression profile in rWAT of genes involved in lipid metabolism. In concrete, they showed higher expression levels of Atgl, the main protein involved in TG mobilization, and of Pparα, the major transcription factor regulating fatty acid oxidation. In adulthood and under HF-diet feeding conditions, CRL animals also maintained higher mRNA expression levels of Atgl than controls. Moreover, at 6 months old, HF-diet-fed CRL females displayed higher expression levels of Cpt1b. Curiously, CRL females but not males, showed lower Fasn and Gpat mRNA levels than controls at weaning, but when animals were 6 months old, CRL rats displayed higher mRNA expression levels of Gpat, under NF-diet conditions, and higher mRNA levels of Fasn, under HF-diet. The greater expression levels of these lipogenic genes in adult CRL animals were not associated with any significant increase in the size of this adipose tissue depot, but could reflect increased capacity of these animals to drive and store excess energy to the adipose tissue. Therefore, differences in the expression levels of Fasn and Gpat between controls and CRL animals seem to be dependent on the age, type of diet and sex of animals.

The liver plays an important role in energy metabolism and is also a target of the peripheral action of leptin and insulin. Concerning insulin signaling, HF-diet-fed female animals in the CRL group, but not males, showed higher mRNA levels of Irs1 compared with their controls, although no significant changes were previously observed at weaning. A similar trend, but not significant, was also found for Insr. Interestingly, 21-day-old CRL pups showed higher Obrb mRNA expression levels, and this pattern was maintained in adult CRL females under HF-diet. The physiological role of peripheral leptin signaling, particularly in liver, and its relative contribution to whole-body energy metabolism, still remain unclear. Peripheral leptin signaling has been considered dispensable for whole body energy homeostasis, since mice lacking peripheral Obrb expression showed no significant alterations in tissue and whole-body energy metabolism (Guo et al. 2007). Nevertheless, leptin action in liver has been shown to attenuate hepatic glucose production and insulin resistance under normal conditions of lean animals, hence contributing to the inhibitory effects of insulin on gluconeogenesis (Brabant et al. 2005). This effect was largely lost in obese animals, which showed decreased

expression of the leptin receptor gene in liver (Brabant et al. 2005). Hence, maintenance of higher Obrb expression levels occurring in CRL females when fed a HF-diet could be related with increased peripheral leptin and insulin sensitivity, as evidenced by the presence of lower circulating levels of leptin, as well as lower L/A ratio and HOMA index. Interestingly, hepatic Obrb mRNA levels in adult animals were correlated negatively with hepatic lipids and with circulating levels of insulin under *ad libitum* feeding conditions (although the latter with a p value of only 0.076). All in all, these results suggest that increased Obrb mRNA levels in liver may be considered beneficial in terms of leptin-to-insulin signaling crosstalk and concerning hepatic lipid storage. Thus, Obrb mRNA levels in liver could be considered as a marker of metabolic health.

Other changes occurring at the gene expression level in liver are also in accordance with the protection of CRL animals against hepatic TG accumulation. That is, CRL pups showed lower mRNA expression levels of Srebp1c than controls. This trend was maintained in adult male animals, particularly under NF-diet conditions. Activation of Srebp1c has been related with increased lipogenesis and has been considered one of the molecular mediators of hepatic steatosis (Browning and Horton 2004). Interestingly, mRNA levels of Srebp1c in liver were positively correlated with hepatic lipid content and also with HOMA index in adult animals (the latter with p=0.070). In addition, adult CRL animals under NF-diet also showed lower expression levels of Gpat, gene that codifies the rate limiting enzyme in TG synthesis (Wendel et al. 2009). Moreover, at weaning, CRL pups showed higher hepatic mRNA expression levels of genes related with lipid mobilization and fatty acid oxidation, Atgl and Cpt1a. ATGL is a key triacylglycerol lipase in the liver, and its overproduction has been associated with reduced steatosis and improved insulin signal transduction in this tissue (Turpin et al. 2011). On the other hand, increased hepatic Cpt1a expression levels may also be a mechanism contributing to the protection of these animals against excessive body weight gain and related metabolic alterations, as evidenced in mice with adenovirus-mediated overexpression of CPT1a in liver (Orellana-Gavalda et al. 2011). However, changes in the expression levels of the genes, Atgl and Cpt1a, by the effect of calorie restriction during lactation, were no longer observed in adulthood.

In conclusion, a very modest maternal calorie restriction of 20% during lactation in rats programs the offspring for better metabolic health in terms of body weight and lipid handling. This condition during lactation determines early adaptations in rWAT and liver, affecting lipogenic and oxidative capacity and increasing their sensitivity to the peripheral effects of

leptin and insulin, which suggests a better control of energy metabolism. These adaptations occurring in early ages are partially maintained in adulthood, and are particularly evident when animals are exposed to an obesogenic environment. Interestingly, among the genes that exhibit changes at the expression level in early ages, Irs1 in rWAT and Obrb and Srebp1c in liver are of relevance because their transcript levels in adult animals were associated with lower hepatic lipid content and improved circulating parameters, thus they could be considered as potential biomarkers of a healthy phenotype. Validation of these biomarkers in human samples, particularly blood cells, which can be easily collected in sufficient quantities by a minimally invasive method, becomes of interest.

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MANUSCRIPT 7

Identification of early transcriptome-based biomarkers related to lipid metabolism in peripheral blood mononuclear cells of rats nutritionally programmed for improved metabolic health

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Title page

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Abstract

Moderate maternal calorie-restriction during lactation protects rat offspring against obesity development in adulthood, due to an improved ability to handle and store excess dietary fuel. We used this model to identify early transcriptome-based biomarkers of metabolic health using peripheral blood mononuclear cells (PBMCs), an easily accessible surrogate tissue, by focusing on molecular markers of lipid handling. Male and female offspring of control and 20% calorie-restricted lactating dams (CRL) were studied. At weaning, a set of pups was killed, and PBMCs were isolated for whole genome microarray analysis. The remaining pups were sacrificed at 6 months of age. CRL gave lower body weight, food intake and fat accumulation, and improved levels of insulin and leptin throughout life, particularly in females. Microarray analysis of weaned rat PBMCs identified 278 genes significantly differentially expressed between control and CRL. Among lipid metabolism-related genes, expression of Cpt1a, Lipe and Star was increased and Fasn, Lrp1 and Rxrb decreased in CRL *versus* control, with changes fully confirmed by qPCR. Among them, Cpt1a, Fasn and Star emerged as particularly interesting. Transcript levels of Cpt1a in PBMCs correlated with their levels in WAT and liver at both ages examined; Fasn expression levels in PBMCs at an early age correlated with their expression levels in WAT; and early changes in Star expression levels in PBMCs correlated with their expression levels in liver and were sustained in adulthood. These findings reveal the possibility of using transcript levels of lipid metabolism-related genes in PBMCs as early biomarkers of metabolic health status.

Keywords: early biomarkers, PBMCs, calorie-restriction, lactation, metabolic programming

Introduction

The increasing prevalence of obesity has become a worldwide phenomenon, affecting both children and adults. Obesity and its related risks can be prevented by lifestyle changes and especially by changes in diet (Perk et al. 2012). Specific diets, foods and food-components can contribute to the development of urgently needed therapeutic and preventive strategies. The development of such nutrition and food based strategies is, however, severely hampered by the lack of predictive biomarkers, especially those that are accessible and quantify health (van Ommen et al. 2009). In order to identify such biomarkers a model is needed that results in changes that reflect the later health status. Such a model is provided by moderate calorie restriction during lactation. Obesity and related pathologies can be programmed by maternal nutrition during the perinatal period (Sullivan and Grove 2010; Pico et al. 2012). While maternal calorie-restriction during pregnancy has been associated with adverse health outcomes in adult offspring (Palou et al. 2012; Palou et al. 2010a), moderate maternal calorie-restriction during lactation in rats has been shown to confer certain protection in the pups against development of obesity and related metabolic alterations associated with high-fat (HF) diet feeding, particularly dyslipidemia, insulin resistance, and hyperleptinemia (Palou et al. 2010b). This provides an animal model that is suited to identify early biomarkers for metabolic health, in terms of a reduced tendency to develop overweight and its associated metabolic complications in adult life.

Suitability of biomarkers for efficacy substantiation requires that they can readily be assessed in humans. Most studies that mechanistically assess effects of diet and foods on health examine tissues such as adipose tissues, muscle or liver which require invasive tissue biopsies (de Mello et al. 2012). Peripheral blood mononuclear cells (PBMCs) provide an attractive alternative that can be assessed in humans, because they can be easily and repeatedly collected in sufficient quantities (de Mello et al. 2008). Gene expression responses of PBMCs have been shown to reflect the liver environment (de Mello et al. 2012), as well as adipose tissue (Caimari et al. 2010b; Caimari et al. 2010a). Therefore, whole genome transcriptome profiling of PBMCs of pups from calorie restricted mothers during lactation may be used to identify early biomarkers, reflecting current and later health of metabolic tissues.

The aim of the current study was to identify early potentially predictive biomarkers of metabolic health by transcriptome profiling of PBMCs. As a model, we used the

offspring of 20% maternal calorie-restricted dams during lactation, which are less prone to obesity development compared to *at libitum* fed controls. We also ascertained i) to what extent the potential markers of optimized health identified at early ages continued to serve as potential markers in adulthood and ii) whether the changes occurring in blood cells reflected the metabolic environment in key tissues. We focused on selected genes of lipid metabolism and assessed these in relevant metabolic tissues, liver and white adipose tissue (WAT), at different ages.

Materials and Methods

Animals and experimental design

The animal protocol followed in this study was reviewed and approved by the Bioethical Committee of the University of the Balearic Islands (Resolution Number 1798. February 18th, 2009) and guidelines for the use and care of laboratory animals of the University were followed.

The study was conducted on male and female Wistar rats from 16 different litters following the protocol described below. All animals were housed under standard conditions, that is, controlled temperature (22 ºC), the normal 12 h light and 12 h dark cycle, free access to tap water and a standard chow diet (3 kcal/g, with 8% calories from fat; Panlab, Barcelona, Spain), unless specified otherwise. Briefly, 16 virgin female Wistar rats (body weight 225 - 260 g) were mated with male rats (Charles River Laboratories, Barcelona, Spain). After mating, each female was placed in an individual cage. On day 1 after delivery, excess pups in each litter were removed aiming for 10 pups per dam (five males and five females, when possible). Dams were assigned to either the control (n=11 dams) or calorie-restricted (n=5 dams) group. Control dams were fed *ad libitum* with standard chow diet (Panlab, Barcelona, Spain), while calorie-restricted dams were fed daily with a 20% calorie-restricted diet throughout lactation, starting on day 1 after delivery until weaning (day 21) as previously described (Palou et al. 2010b). During the lactating period, body weight of male and female offspring of control and calorie-restricted dams (control and CRL, respectively) was followed.

At weaning, a set of animals made up of 24 pups from control (12 males and 12 females) and 24 from CRL (12 males and 12 females) group were sacrificed by decapitation under *ad libitum* feeding conditions. One half of pups (n=6/group) were

use to obtain different WAT depots (inguinal and retroperitoneal; iWAT and rWAT, respectively) and liver (which were rapidly removed, frozen in liquid nitrogen and stored at -70ºC until RNA analysis), as well as trunk blood samples (for peripheral blood mononuclear cells (PBMCs) isolation, as described in the next section). From the other half of pups (n=6/group), blood samples were collected in heparinized containers and plasma was obtained by centrifugation at 700 g for 10 min.

Another set of animals, 28 control pups (12 males and 16 females) and 26 CRL pups (14 males and 12 females), were kept alive. They were placed two per cage, paired with another animal of the same group, and fed *ad libitum* with a normal-fat (NF) diet (3.8 kcal/g, 10% calories from fat, Research Diets, Inc., NJ, USA) until the age of 6 months. Body weight and food intake of those animals were followed. Moreover, at the age of 6 months (prior to sacrifice), blood samples were collected at fed state and after 12 h fasting to obtain plasma. All the animals were decapitated under *ad libitum* feeding conditions at the age of 6 months, and samples of trunk blood for PBMCs isolation were collected. Body length (from the tip of the nose to the anus) and body composition (by EchoMRI-700TM, Echo Medical Systems, LLC., TX, USA) were measured in control and CRL animals without anesthesia when animals were 21 days and 6 months old.

Measurement of circulating parameters under fed/fasting conditions, and calculation of the homeostatic model assessment for insulin resistance (HOMA-IR) at different ages

Blood samples collected at the ages of 21 days (under *ad libitum* feeding conditions) and 6 months (under *ad libitum* and 12 h fasting conditions) were used for analysis of circulating parameters. Blood glucose concentration was measured by Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Peripheral hormones were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits: insulin concentration was determined using a rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden) and leptin with QuantikineTM Mouse Leptin Inmunoassay (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Plasma non-esterified fatty acid (NEFA) and triglyceride levels (TG) were determined with a commercial enzymatic colorimetric kits (Wako Chemicals GmbH, Neuss, Germany and Triglyceride (INT) 20, Sigma Diagnostics, St Louis, MO, USA, respectively), following standard procedures. The homeostatic model assessment for insulin resistance (HOMA-IR) was used to assess insulin resistance. It is calculated from fasting insulin and

glucose concentration using the formula of Matthews et al. (Matthews et al. 1985): HOMA-IR=fasting glucose (mmol/liter) x fasting insulin (mU/liter)/22.5.

PBMC isolation

Trunk blood samples of control and CRL rats collected (at the age of 21 days and 6 months) under *ad libitum* feeding conditions, were used to isolate PBMCs. Peripheral blood samples were collected using heparin in NaCl (0.9%) as anticoagulant, and then diluted with an equal volume of balanced salt solution. PBMCs were immediately isolated by Ficoll density-gradient separation according to the instructions of the manufacturer (GE Healthcare Bio Sciences, Barcelona, Spain).

Total RNA isolation

Total RNA was extracted from iWAT, rWAT, liver and PBMCs of control and CRL animals by EZNA® TOTAL RNA kit I (Omega Bio-Tek Inc., Norcross, GA, USA) following the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Techonologies, Ins., Wilmington, DE) and its integrity confirmed using 1% agarose gel electrophoresis (for iWAT, rWAT and liver).

Microarray processing

For microarray analysis, RNA from PBMC samples obtained from male and female offspring of controls and CRL animals at the age of 21 days were used (n=6/group). RNA samples were analyzed on Agilent 2100 Bioanalyzer with RNA 6000 Nano chips (Agilent Technologies, South Queensferry, United Kingdom). To assure the high quality of RNA, all samples used for microarrays had a RIN number ≥ 8. Then, 0.04 µg of RNA from each sample was reverse transcribed to complementary DNA (cDNA) using the Agilent Low Input Quick Amp Labeling kit (Agilent Technologies, Inc., CA, USA), according to the manufacturer's protocol. Then, half of the cDNA sample (10 µl) was used for the linear amplification of RNA and labeling with cyanine-3 (Cy3) or Cy5. For these reactions, half of the amounts indicated by the manufacturer were used (van Schothorst et al. 2007). Transcription and labeling were carried out at 40 °C for 2 h. Then, the labeled and amplified cRNA samples were purified using Qiagen Rneasy MiniSpin columns (Qiagen, Venlo, the Netherlands). The incorporation of dyes and

cRNA concentration was measured using the "microarray measurement mode" of the NanoDrop ND 1000 spectrophotometer (NanoDrop Techonologies, Ins., Wilmington, DE). Each sample containing 600 ng of cRNA labeled with Cy5 and 600 ng of Cy3 pool were hybridized on 4x44K G4131F rat whole genome Agilent microarrays (Agilent Technologies, Inc., Santa Clara, CA, USA) for 17 h at 65 °C in hybridization chambers in an oven rotating at 10 rpm (Agilent Technologies). After hybridization, the arrays were washed with "GE wash buffer 2" for 1 min at 37 °C, followed by acetonitrile for 1 min at room temperature, and finally with a solution for stabilization and drying for 30 s at room temperature, according to the manufacturer's protocol (Agilent Technologies).

Microarray data analysis

The arrays were scanned with an Agilent Microarray Scanner (Agilent Technologies). Scanned images were examined for visible defects and proper grid alignment. The intensities of the signals from each spot were quantified, and the raw data were extracted using Feature Extraction Software version 10.5.1.1 (Agilent Technologies, Inc., Santa Clara, CA, USA). Quality control was performed for each of the arrays using LimmaGUI package in R from Bioconductor Software version 2.1. Only one of the arrays did not pass quality control based on MA plot and signal intensity distribution (Allison et al. 2006). Thus, in total, dataset from 23 arrays passed to the next step of analysis. Data were exported into GeneMaths XT 2.12 (Applied Mathematics, Sint-Martens-Latem, Belgium) for background correction and normalization. Locally weighted linear regression (lowess) analysis was chosen as a normalization method, which enables intensity-dependent effects in the log2 (ratio) values to be removed (Yang et al. 2002). Then, the values were converted to log2 values and the target samples (Cy5) intensities were normalized against the intensities of reference samples (Cy3), as described previously (Pellis et al. 2003). Target signals with an average intensity lower than twofold above average background were discarded to increase accuracy of the data. Correction for multiple testing was not applied, as these corrections are often too strict to identify small effects which are usually observed in nutritional studies (Pellis et al. 2003). Validity of data was assured by checking biological plausibility and by independent analysis by RT-qPCR (see below) in PBMCs and two metabolic tissues (liver and WAT).

To search for biomarkers of metabolic interest in both sexes, two-way analysis of variance (ANOVA) with factors of sex and experimental group was performed. The threshold of significance for this statistical test was set at $p \leq 0.01$. Moreover, fold change (FC) calculation between both groups of animals (CRL *vs* control animals) was performed; FC equals the expression ratio between CRL and controls in the case of increase, or equals −1/ratio in the case of decrease. The analyses were performed with SPSS for Windows (SPSS version 19.0, Chicago, IL). Subsequently, a statistically generated list of genes was manually analyzed in regard to their biological information, obtained with the use of available databases (Genecards, KEGG, NCBI, Reactome, UniProt, USCN, WikiPathways) based on key biological domains, such as molecular function and biological process. Some of these processes overlapped, thus they were collected, renamed and all the unique genes were assigned into several biological processes according to their function.

Real-time quantitative RT-polymerase chain reaction (RT-qPCR) analysis

To validate microarray data, mRNA expression levels of apolipoprotein B48 receptor (Apob48r), carnitine palmitoyltransferase 1 alpha (Cpt1a), fatty acid synthase (Fasn), hormone-sensitive lipase (Lipe), low density lipoprotein receptor-related protein 1 (Lrp1), phosphate cytidylyltransferase 2, ethanolamine (Pcyt2), retinoid X receptor beta (Rxrb), sortilin-related receptor, LDLR class A repeats-containing (Sorl1) and steroidogenic acute regulatory protein (Star) were measured by RT-qPCR in PBMC RNA samples of control and CRL animals. Additionally, RT-qPCR was performed to analyze mRNA expression of aforementioned genes in PBMCs at the age of 6 months, and in iWAT, rWAT and liver, at the age of 21 days and 6 months. Regarding Star, its mRNA levels were only analyzed in liver, as its expression has not been described in WAT. We did not analyze Apob48r or Sorl1 expression either in liver nor in WAT, as both genes are expressed mainly in blood and neural cells.

For RT-qPCR analysis, 0.05 µg of PBMC total RNA was used for reverse transcription by using iScript^{TM} cDNA synthesis kit (Bio-Rad Laboratories, S.A., Madrid, Spain) according to the manufacturer's protocol. For iWAT, rWAT and liver, 0.25 µg of total RNA (in a final volume of 5 µl) was firstly denatured at 65 ºC for 10 min, and then reverse transcribed to cDNA with MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20 ºC for 15 min, at 42 ºC for 30 min, with a final step of 5 min at 95

ºC in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain).

Real-time PCR was performed using the Applied Biosystems StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) with Power SYBER Green PCR Master Mix (Applied Biosystems, CA, USA). Each PCR was performed from 1/5 dilution of the cDNA product and forward and reverse primers (5 µM each). Primer sequences and products for the different genes are described in Table 1.

Table 1. Nucleotide sequences of primers and amplicon size used for RT-qPCR analysis of mRNA expression levels of selected genes in PBMCs, iWAT, rWAT and liver samples.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon (bp)
β-actin	*TACAGCTTCACCACCACAGC*	*TCTCCAGGGAGGAAGAGGAT*	120
Apob48r	*GGGCTACATCAGGCTTTGAG*	*TTCCTCCCCTACAACCTTCC*	150
Cpt1a	*GCTCGCACATTACAAGGACAT*	*TGGACACCACATAGAGGCAG*	250
Fasn	*CGGCGAGTCTATGCCACTAT*	*ACACAGGGACCGAGTAAT*	222
Gdi-1	*CCGCACAAGGCAAATACATC*	*GACTCTCTGAACCGTCATCAA*	210
Lipe	*TCACGCTACATAAAGGCTGCT*	*CCACCCGTAAAGAGGGAACT*	169
Lrp1	*GAGCAGGTTGTCAGTCAGCA*	*TAGGGTTTCCGATTTCCACA*	187
Pcyt2	*CCGACAGGGATGGGTCTG*	*TGGCTTCCTTCTTCTGATTCC*	156
Rxrb	*CCCTTCCCAGTCATCAGTTC*	*GGTGGCTTCACATCTTCAGG*	152
Sorl1	*CACCGTCTCATTGTCAGCAC*	*ATCTCGTAGCCCCTGGTTTC*	123
Star	*GGGTGGATGGGTCAGGTC*	*CTGCTGGCTTTCCTTCTTCC*	168
Tbp	*ACCCTTCACCAATGACTCCTATG*	*ATGATGACTGCAGCAAATCGC*	190

Abbreviations: apolipoprotein B48 receptor (Apob48r); carnitine palmitoyltransferase 1alpha (Cpt1a); fatty acid synthase (Fasn); GDP dissociation inhibitor 1 (Gdi-1); hormone-sensitive lipase (Lipe); low density lipoprotein receptor-related protein 1 (Lrp1); phosphate cytidylyltransferase 2, ethanolamine (Pcyt2); retinoid X receptor beta (Rxrb); sortilin-related receptor, LDLR class A repeats-containing (Sorl1); steroidogenic acute regulatory protein (Star) and TATA-box binding protein (Tbp)

All primers were purchased from Sigma Genosys (Sigma Aldrich Química SA, Madrid, Spain). After an initial Taq activation at 95 ºC for 10 min, PCR was performed using 40 two-temperature cycles with the following cycling conditions: 95 ºC for 15s and 60 ºC for 1 min. To verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The values for the threshold (Ct) were calculated by the instrument's software (StepOne Software v2.2.2), and the relative expression of each mRNA was calculated as a percentage of male control rats, using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001) with β-actin, GDP dissociation inhibitor 1 (Gdi-1) and TATA-Box Binding Protein (Tbp) as reference genes.

Statistical analysis

All data were expressed as mean ± S.E.M. The statistical analysis of microarray data has been described in details in the section referred to microarray data analysis. Multiple comparisons were assessed by ANOVA repeated measures and two-way ANOVA to determine the effects of different factors (moderate maternal calorie-restriction during lactation and sex). Single comparisons between groups were assessed by Student's *t* test and Paired *t* test. Person´s correlation coefficient was used to determine the association between the expression pattern of lipid metabolism-related genes in PBMCs and other tissues. $P<0.05$ was the threshold of significance, unless stated. The analyses were performed with SPSS for Windows (SPSS version 19.0, Chicago, IL).

Results

Phenotypic characteristics and blood parameters throughout life

Maternal calorie-restriction of 20% during lactation resulted in lower body weight of both male and female offspring in comparison to control animals (Figure 1A). This effect was significant from the age of 5 days and was persistent during the whole study period (6 months) ($p<0.05$; two-way ANOVA). When animals were 6-month-old CRL male and female rats weighed 7.8% and 11.4% less than their controls, respectively (Table 2A).

Notably, cumulative calorie intake of animals from weaning (day 21) until the age of 6 months (Figure 1B) was significantly lower in CRL animals compared with controls (3.0% and 7.5% less, in males and females, respectively; $p<0.05$; two-way ANOVA). This may explain, at least in part, the lower body weight occurring in CRL animals, particularly females.

Other morphological traits of young and adult offspring are summarized in Table 2A. At weaning (21d), both male and female CRL pups showed lower body length than the controls ($p<0.05$; two-way ANOVA), but no significant differences were found in adult animals in either sex. In addition, at the age of 21 days CRL male and female animals showed lower body fat content (relative to their body weight) than control animals, as well as lower weight of inguinal and retroperitoneal WAT depots and of liver ($p<0.05$; two-way ANOVA). In turn, at the age of 6 months, female CRL animals, but not males, presented lower body fat content, as well as lower weight of iWAT and rWAT than

controls (interactive effect between sex and calorie-restriction, $p<0.05$; two-way ANOVA), and both CRL males and females displayed lower weight of liver ($p<0.05$; two-way ANOVA).

Plasma circulating parameters of control and CRL animals under *ad libitum* feeding conditions at weaning (21 days), as well as under *ad libitum* and 12 h fasting conditions at the age of 6 months are shown in Table 2B. At weaning, CRL pups displayed significantly lower plasma levels of glucose, insulin and leptin relative to their controls ($p<0.05$; two-way ANOVA). No differences were found in NEFA and TG levels between control and CRL pups.

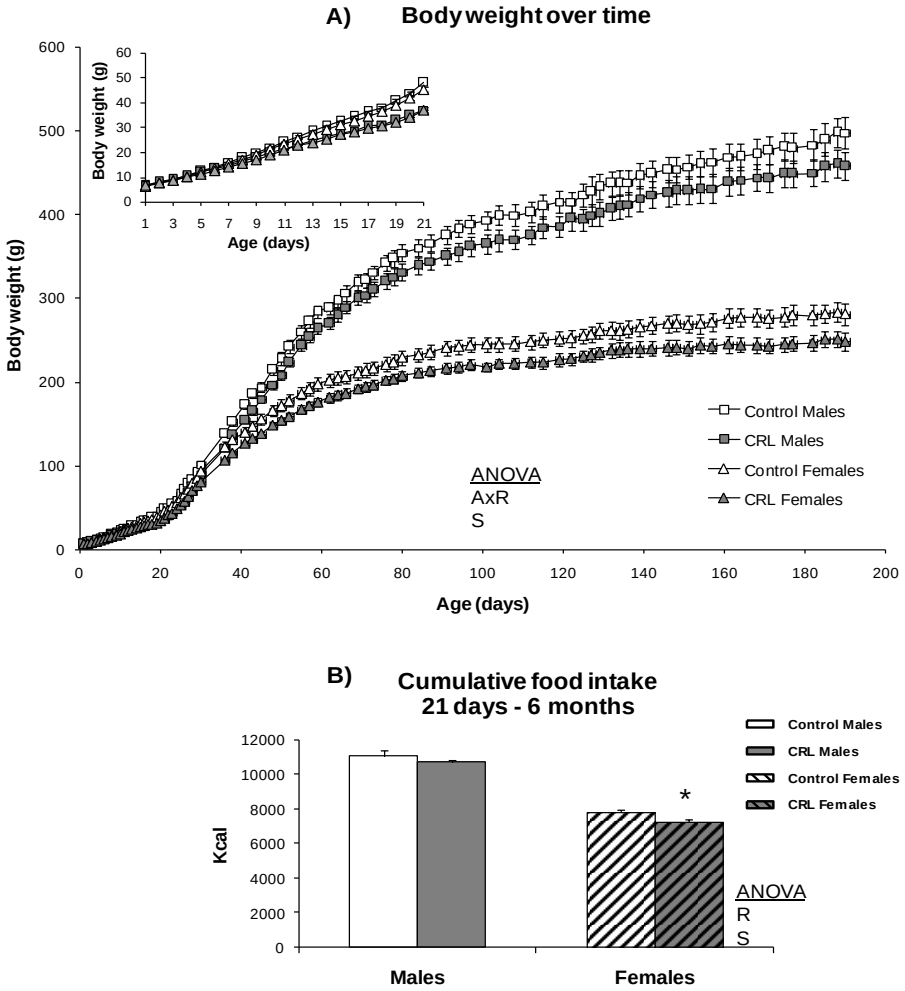

Figure 1A. Body weight with time of male and female offspring of control and calorie-restricted dams during lactation (CRL) from day 1 until day 190 of age. The inset details body weight throughout the lactating period (from day 1 until day 21 of age). Data are mean ± S.E.M. (n=12-16 animals/group). Statistics: AxR, interaction between age and calorie-restriction during lactation; S, effect of sex ($p<0.05$; ANOVA repeated measures). **B**. Cumulative food intake (Kcal) of male and female offspring of controls and calorie-restricted dams during lactation (CRL) fed *ad libitum* with normal-fat diet from 21 days until the age of 6 months. Data are mean ± S.E.M. (n=12-16 animals/group until the age of 21 days; n=6-8 animals/group until the age of 190 days). Statistics: R, effect of maternal calorie-restriction during lactation; S, effect of sex ($p<0.05$; two-way ANOVA). *, CRL *vs* Controls ($p<0.05$; Student's *t* test).

Table 2. Morphological traits (A) and circulating parameters (B) in male and female offspring of control and calorie-restricted dams during lactation (CRL) at weaning (21-day-old) and at the age of 6 months.

			Males		Females		
			Control	**CRL**	**Control**	**CRL**	**ANOVA**
A) Morphological traits							
21 days	Body weight (g)		48.1 ± 2.1	37.1 ± 0.8 *	45.4 ± 1.1	36.9 ± 0.5 *	R
	Body length (cm)		11.5 ± 0.2	10.8 ± 0.2*	11.0 ± 0.2	10.4 ± 0.1*	R, S
	Body fat (%)		10.0 ± 0.4	8.68 ± 0.26*	10.5 ± 0.3	8.87 ± 0.19*	R
	iWAT (mg)		294 ± 35	187 ± 19*	307 ± 33	186± 26*	R
	rWAT (mg)		69.6 ± 9.9	40.1 ± 4.9*	50.5 ± 3.7	24.9 ± 3.2 *	R
	Liver (g)		1.68 ± 0.05	1.25 ± 0.08*	1.76 ± 0.06	1.29 ± 0.03 *	R
6 months	Body weight (g)		498 ± 19	459 ± 17	281 ± 12	249 ± 11	R, S
	Body length (cm)		24.2 ± 0.5	24.0 ± 0.3	20.8 ± 0.3	20.3 ± 0.2	S
	Body fat (%)		20.7 ± 2.2	22.1 ± 1.6	22.1 ± 2.3	14.5 ± 1.2 *	RxS
	iWAT (g)		9.95 ± 1.20	10.7 ± 0.7	3.93 ± 0.37	2.14 ± 0.45 *	RxS
	rWAT (g)		12.8 ± 1.7	12.4 ± 1.0	4.53 ± 0.67	2.16 ± 0.23 *	RxS
	Liver (g)		16.0 ± 0.9	13.4 ± 0.6 *	8.13 ± 0.47	7.35 ± 0.28	S, R
B) Circulating parameters							
21 days	Glucose (mg/dL)	Fed	137 ± 7	120 ± 3*	141 ± 5	116 ± 4*	R
	Insulin (ng/L)	Fed	0.111 ± 0.042	0.044 ± 0.010	0.105 ± 0.020	0.037 ± 0.004 *	R
	Leptin (ng/L)	Fed	0.941 ± 0.154	0.408 ± 0.045 *	1.05 ± 0.11	0.585 ± 0.162*	R
	NEFA (nM)	Fed	1.46 ± 0.13	1.50 ± 0.14	1.37 ± 0.19	1.59 ± 0.13	
	TG (mg/mL)	Fed	1.02 ± 0.14	0.893 ± 0.130	0.938 ± 0.094	0.851 ± 0.042	
6 months	Glucose (mg/dL)	Fed	108 ± 3	107 ± 6	105 ± 4	102 ± 4	
		Fasting	98 ± 8	95 ± 3#	102 ± 3	90 ± 1 *,#	R (p=0.059)
	Insulin (ng/L)	Fed	2.77 ± 0.94	1.44 ± 0.26	0.835 ± 0.102	0.459 ± 0.050*	R (p=0.069), S
		Fasting	0.920 ± 0.379 #	1.06 ± 0.24 #	0.558 ± 0.121 #	0.287 ± 0.047 #	
	HOMA-IR	Fasting	5.37 ± 2.38	5.77 ± 1.01	3.33 ± 0.75	1.67 ± 0.32	S
	Leptin (ng/L)	Fed	15.8 ± 2.2	13.4 ± 1.1	6.07 ± 0.94	1.74 ± 0.19 *	RxS
	NEFA (nM)	Fed	0.851 ± 0.152	0.910 ± 0.056	1.33 ± 0.24	0.866 ± 0.086	
	TG (mg/mL)	Fed	1.61 ± 0.08	1.40 ± 0.19	1.02 ± 0.12	0.920 ± 0.073	S

Data are mean ± S.E.M. (n=6-8 animals/group) of male and female offspring of control and calorie-restricted dams during lactation (CRL), under *ad libitum* feeding conditions (Fed) and after 12 h fasting (Fasting). Statistics: R, effect of maternal calorie-restriction during lactation; F, effect of fasting conditions; S, effect of sex; FxR, interaction between fasting conditions and maternal calorie-restriction during lactation; RxS, interaction between maternal calorie-restriction during lactation and sex($p<0.05$; ANOVA repeated measures).*, CRL *vs* Controls ($p<0.05$; Student's *t* test); #, fasting *vs* fed conditions ($p<0.05$; Paired *t* test). Abbreviations: iWAT, inguinal white adipose tissue; rWAT, retroperitoneal white adipose tissue; NEFA, non-esterified fatty acid; TG, triglycerides.

At the age of 6 months, a tendency to lower glucose and insulin levels (under fasting and fed conditions, respectively) was found in CRL animals *versus* controls ($p=0.059$ and $p=0.069$, respectively; two-way ANOVA). The decrease was particularly pronounced and statistically significant in females ($p<0.05$; Student's *t* test). CRL

females also showed lower leptin levels than their controls (the latter only under fed conditions) ($p<0.05$; Student's *t* test), but no significant differences were found between males. Fasting conditions resulted in a significant decrease in glucose levels, only in CRL animals, and in insulin levels in the different groups of animals ($p<0.05$; Paired *t* test). No significant differences were found between control and CRL animals concerning HOMA-IR index, although CRL female animals showed a tendency to lower values than controls at 6 months ($p=0.076$; Student's *t* test). No significant differences were found concerning circulating NEFA and TG levels between control and CRL adult animals.

Lipid metabolism-related gene expression in PBMCs of pups at the age of 21 days based on whole-genome microarray analysis

In our microarray analysis, 45,018 probes were tested. Of them, those having an expression value of twice above the background (22,920) were further taken into account and normalized. In total 310 probes were found to be significantly different between control and CRL animals ($p\leq0.01$; two-way ANOVA). Removal of duplicates resulted in 278 unique genes. Using available databases (Genecards, KEGG, NCBI, Reactome, UniProt, USCN, WikiPathways), these genes were classified into several biological processes according to their function. 111 genes were unknown, thus were not included in any of the processes. From the remaining 167 known genes, 113 exhibited down-regulation and 54 up-regulation. As shown in Figure 2, the processes with the highest number of genes differentially expressed were related to immune system, signaling, cell turnover, transcription machinery and transport (28, 24, 19, 16 and 12 genes, respectively). Other processes with a notable number of genes were related to metabolism of proteins, carbohydrates and lipids (11, 11 and 9, respectively). The remaining genes were related with the nervous system, redox metabolism, cytoskeleton, neural signaling, blood, cell communication, central metabolism, sensory perception, and food intake control (9 or less genes involved in each of the processes).

Subsequently, genes involved in lipid metabolism were analyzed in detail (Table 3). Of the 9 genes involved in this process (Apob48r, Cpt1a, Fasn, Lipe, Lrp1, Pcyt2, Rxrb, Sorl1, Star), 6 showed down-regulation and 3 up-regulation. Down-regulated genes were involved in lipogenesis/lipolysis, glycerophospholipid biosynthesis and low

density lipoprotein uptake. Up-regulated genes were involved in β-oxidation, lipolysis and cholesterol transport.

Confirmation of microarray results by RT-qPCR

To confirm gene array findings and to test whether the changes were consistent, RT-qPCR analysis of genes involved in the process of lipid metabolism was performed on the same RNA samples of 21-day-old male and female control and CRL animals. Genes chosen for confirmation were: Fasn (lipogenesis), Rxrb (lipogenesis/lipolysis), Cpt1a (β-oxidation), and Lipe (lipolysis); Apob48r, Lrp1, and Sorl1 (all involved in lipoprotein uptake); Pcyt2 (glycerophospholipid biosynthesis); Star (cholesterol transport and uptake).

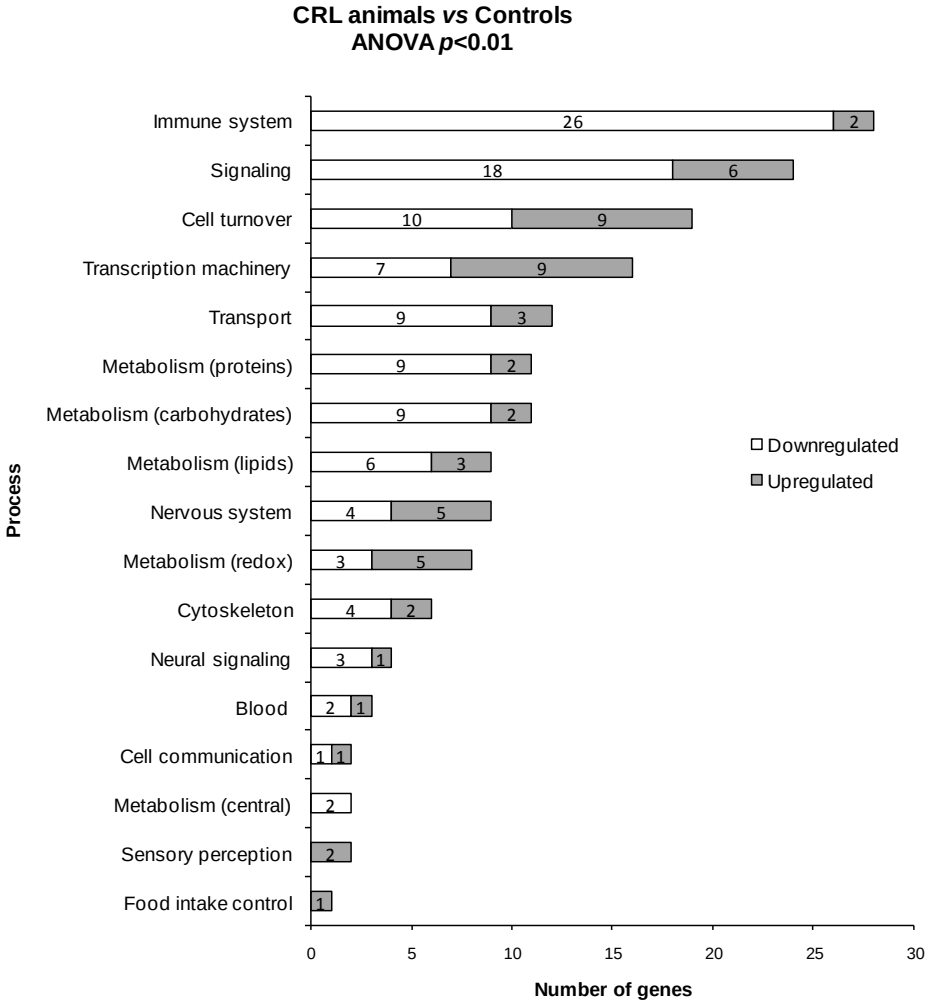

Figure 2. Classification into biological processes of the genes differentially expressed in PBMCs samples of male and female offspring of control and calorie-restricted dams during lactation (CRL) at the age of 21 days. Statistical analysis was performed by considering males and females as a whole ($p \leq 0.01$; two-way ANOVA). The number of genes down- or up-regulated is indicated for each group of genes.

Table 3. Microarray data of genes involved in lipid metabolism process and their validation by RT-qPCR in PBMCs samples of male and female offspring of control and calorie-restricted dams during lactation (CRL) at the age of 21 days.

Related process	Gene name	Gene symbol	Sequence ID	*Microarray*			qPCR		
				p-value*	Fold change		*p*-value**	Fold change	
					Males	Females		Males	Females
Low density lipoproteins uptake	Apolipoprotein B48 receptor	Apob48r	NM_001109154	0.007	-1.14	-1.29	0.667	+1.00	+1.03
β-oxidation	Carnitine palmitoyltransferase 1 alpha, liver	Cpt1a	NM_031559	0.004	+1.22	+1.15	0.002	+1.17	+1.13
Lipogenesis	Fatty acid synthase	Fasn	NM_017332	0.002	-1.11	-1.25	0.025	-1.19	-1.03
Lipolysis	Lipase, hormone sensitive	Lipe	NM_012859	0.01	+1.14	+1.10	0.004	+1.09	+1.07
Low density lipoproteins uptake	Low density lipoprotein receptor-related protein 1	Lrp1	NM_001130490	0.007	-1.15	-1.04	0.038	-1.05	-1.09
Glycerophospholipid biosynthesis	Phosphate cytidylyltransferase 2, ethanolamine	Pcyt2	NM_053568	0.000	-1.07	-1.24	0.166	-1.00	-1.10
Lipogenesis/lipolysis	Retinoid X receptor beta	Rxrb	NM_206849	0.002	-1.33	-1.17	0.026	-1.10	-1.05
Low density lipoproteins uptake	Sortilin-related receptor, LDLR class A repeats-containing	Sorl1	NM_053519	0.002	-1.08	-1.34	0.238	-1.07	-1.03
Cholesterol transport and uptake	Steroidogenic acute regulatory protein	Star	NM_031558	0.006	+1.11	+1.09	0.047	+1.09	+1.10

p-value * of microarray data and *p*-value ** of RT-qPCR data for statistical analysis (two-way ANOVA). Threshold of significance was set at $p \leq 0.01$ and $p < 0.05$ for microarray and RT-qPCR data. Ratios indicating fold changes in experimental group (CRL *vs* Controls) are presented for microarray and RT-qPCR data. +, indicates upregulation; -, downregulation in CRL animals of both sexes.

RT-qPCR analysis confirmed most of the microarray data (Table 3), as differences in the expression levels of Cpt1a, Lipe, Star and Fasn, Lrp1 and Rxrb between control and CRL animals reached statistical significe ($p<0.05$; two-way ANOVA) and followed the same pattern of up- and down-regulation as observed in the microarray analysis. Moreover, fold changes of those genes were similar using both techniques. Although RT-qPCR analysis of Pcyt2 did not reveal significant differences between control and CRL animals, single comparison between groups revealed that CRL females exhibited a trend to lower Pcyt2 mRNA levels relative to their controls ($p=0.068$; Student's *t* test). Differences for Apob48 and Sorl1 identified using the microarrays could not be confirmed by RT-qPCR analysis.

Comparison of mRNA expression levels of genes involved in lipid metabolism in PBMCs with the expression measured in different tissues (iWAT, rWAT and liver) at the age of 21 days

To determine whether changes in mRNA levels of genes involved in lipid metabolism due to moderate maternal calorie-restriction during lactation observed in PBMCs of 21-day-old pups reflected the changes occurring in other tissues involved in lipid metabolism, we analyzed their mRNA expression levels in liver and in iWAT and rWAT, representative of subcutaneous and internal adipose tissue depots, respectively (Figure 3). In accordance with findings in PBMCs, CRL animals showed higher Cpt1a mRNA levels in both WAT depots and in liver compared with controls ($p<0.05$; two-way ANOVA). Fasn expression levels in iWAT and rWAT were also significantly decreased in CRL animals ($p<0.05$; two-way ANOVA), in agreement with changes occurring in PBMCs. No significant changes between control and CRL animals were found in liver, although a trend to lower Fasn mRNA levels was observed in CRL females ($p=0.066$; Student's *t* test). Differences found in PBMCs for Lipe mRNA expression levels were also found in iWAT, and for Star in liver ($p<0.05$; two-way ANOVA). Concerning Pcyt2, the trend to lower mRNA levels occurring in CRL female animals with respect to their controls was also observed in both WAT depots and in liver by Student's *t* test.

However, changes found for Rxrb in PBMCs between control and CRL animals were not related to changes in WAT or liver, where no changes in the expression levels of this gene could be shown as an effect of maternal calorie-restriction during lactation.

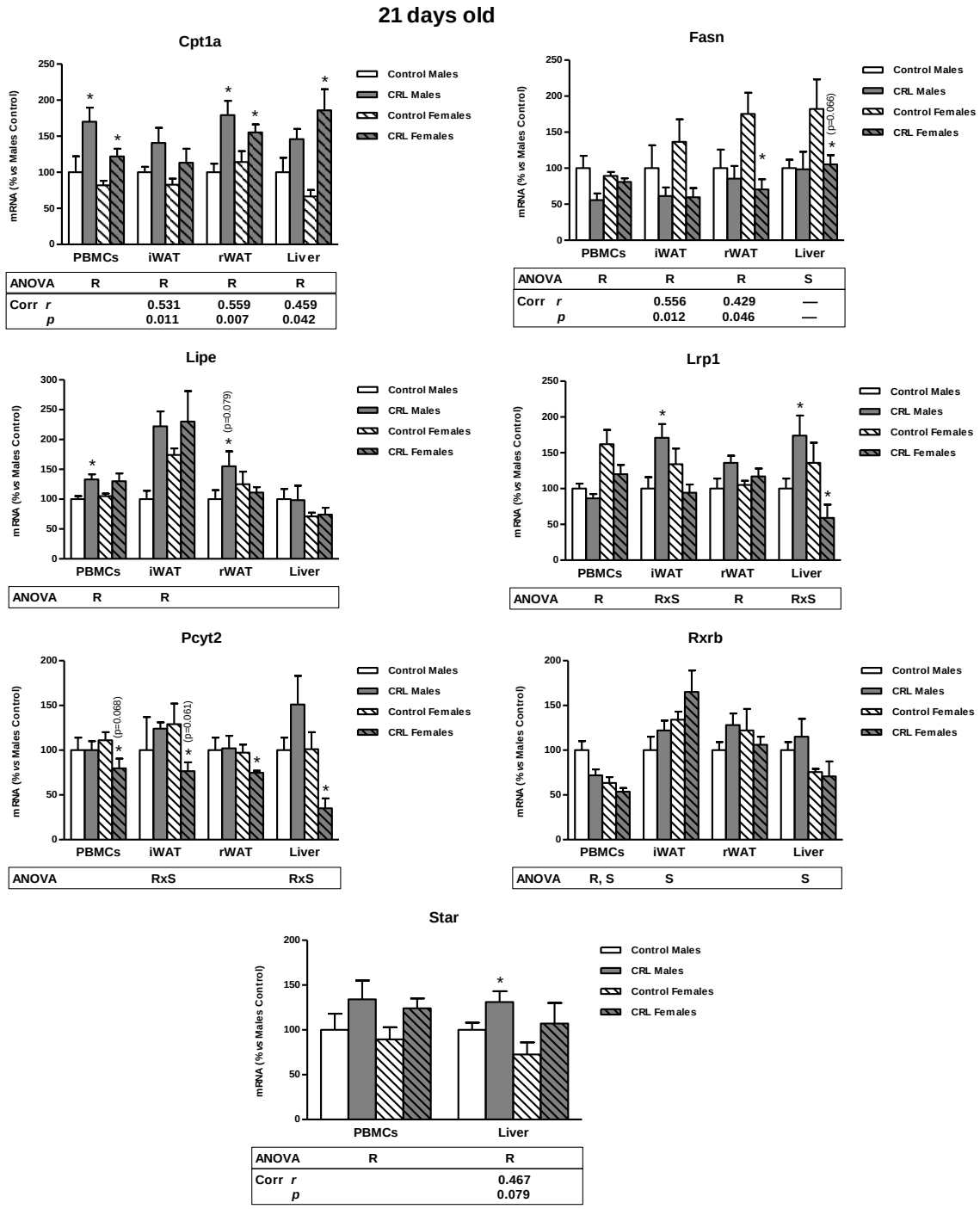

Figure 3. Comparison of mRNA expression levels of lipid metabolism-related genes in PBMCs, with the expression determined in inguinal and retroperitoneal WAT and liver of male and female offspring of control and calorie-restricted dams during lactation (CRL) at the age of 21 days. mRNA levels were measured by RT-qPCR and expressed as a percentage of the mean value of males control. Data are mean ± S.E.M (n=6-7 animals/group). Statistics: R, effect of maternal calorie-restriction during lactation; S, effect of sex; RxS, interaction between the effect of maternal calorie-restriction during lactation and the effect of sex ($p<0.05$; two-way ANOVA). *, CRL *vs* Controls ($p<0.05$; Student's *t* test); Corr, consistent positive correlations between mRNA expression levels of selected genes involved in lipid metabolism in PBMCs and mRNA expression levels of those genes in iWAT, rWAT and liver of male and female offspring of control and calorie-restricted dams during lactation (CRL) at the age of 21 days; *r*, Pearson's correlation coefficient; *p*-value of the genetic correlation ($p<0.05$); – indicates no correlations.

Finally, changes for Lrp1 in PBMCs followed the same trend in iWAT and liver (however only for females), but were in the opposite direction to those found in rWAT ($p<0.05$; two-way ANOVA).

To evaluate how closely the changes in the lipid metabolism-related gene expression levels in PBMCs indicate those occurring in liver, iWAT and rWAT, correlation tests on the corresponding genes were performed (correlation values are indicated in Figure 3). Notably, Cpt1 mRNA levels in PBMCs were positively correlated with their expression levels in iWAT, rWAT and liver; and also Fasn mRNA levels in PBMCs correlated positively with their expression levels in iWAT and rWAT. Star mRNA levels in PBMCs were slightly correlated with Star mRNA levels in liver. On the other hand, no associations were found in relative mRNA expression responses of the other genes studied in PBMCs (Lipe, Lrp1, Pcyt2 and Rxrb) with those in WAT or liver (data not shown).

mRNA expression levels of selected genes involved in lipid metabolism in PBMCs at the age of 6 months

We also examined how the mRNA pattern of PBMC lipid metabolism-related genes, which were significantly altered at the age of 21 days in CRL rats, behaved in adult animals (Table 4). Our results show that overexpressed levels of Star in young CRL males and females were maintained in adulthood, although without reaching statistical significance ($p<0.077$; two-way ANOVA). Changes found for Fasn expression levels in young animals were only preserved in adulthood for females ($p<0.05$; Student's *t* test; interactive effect between moderate maternal calorie-restriction during lactation and sex, $p<0.05$; two-way ANOVA). No changes in mRNA levels of Apob48r, Cpt1a, Lipe, Lrp1, Pcyt2 and Sorl1 were observed between control and CRL animals, and, contrary to what was found in young animals, *Cpt1a* mRNA levels were down-regulated in CRL females relative to their controls ($p<0.05$; Student's *t* test).

Table 4. mRNA expression levels of genes involved in lipid metabolism in PBMCs samples of male and female offspring of control and calorie-restricted dams during lactation (CRL) at the age of 6 months.

	Males		Females		
	Control	**CRL**	**Control**	**CRL**	**ANOVA**
Apob48r	100 ± 12	101 ± 12	100 ± 23	110 ± 20	
Cpt1a	100 ± 5	102 ± 8	114 ± 12	84.4 ± 3.6 *	
Fasn	100 ± 8	109 ± 8	126 ± 4	99.7 ± 4.8 *	RxS
Lipe	100 ± 13	107 ± 12	91.9 ± 5.2	92.4 ± 12.4	
Lrp1	100 ± 25	139 ± 39	73.8 ± 13.5	110 ± 13.2	
Pcyt2	100 ± 19	121 ± 28	84.1 ± 8.8	109 ± 22	
Rxrb	100 ± 6	115 ± 6	125 ± 10	103 ± 4	RxS
Sorl1	100 ± 21	96.5 ± 16.1	75.7 ± 2.5	103 ± 18	
Star	100 ± 15	137 ± 22	120 ± 10	167 ± 33	R (p=0.077)

mRNA levels were measured under *ad libitum* feeding conditions at the age of 6 months by RT-qPCR, and expressed as a percentage of the mean value of males control. Data are mean ± S.E.M (n=6-8 animals/group). Statistics: R, effect of maternal calorie-restriction during lactation; RxS, an interaction between the effect of maternal calorie-restriction during lactation and the effect of sex (p<0.05; two-way ANOVA). *, CRL *vs* Controls (p<0.05; Student's *t* test).

Comparison of mRNA expression levels of selected genes involved in lipid metabolism in PBMCs with the expression measured in different tissues (iWAT, rWAT and liver) at the age of 6 months

We next determined if the genes showing differential expression between control and CRL adult animals in PBMCs, Fasn, Cpt1a and Star, also showed this in both fat depots (iWAT and rWAT) and in liver at an older age. We also included Rxrb, because its expression levels in control and CRL animals showed different patterns dependent on the sex of animals (Figure 4). Changes in Cpt1a mRNA levels observed in PBMCs of adult rats reflected those found in rWAT and liver (interactive effect between sex and calorie-restriction, p<0.05; two-way ANOVA), although in rWAT changes did not reach statistical significance. Concerning Rxrb, its mRNA levels in rWAT were differentially expressed in CRL animals depending on sex, the same as observed in PBMCs (interactive effect between sex and calorie-restriction, p<0.05; two-way ANOVA). A similar trend was also found in iWAT for females (p<0.05; Student's *t* test). Star expression profile observed in PBMCs was somewhat reflected in the liver,

although without reaching statistical significance. Regarding Fasn, changes observed at mRNA levels of adult CRL animals with respect to controls in PBMCs were not reflected either in fat depots or in liver.

As in young animals, we assessed the strength of association between the expression levels of these genes in PBMCs with those in iWAT, rWAT and liver of adult animals (Figure 4) by correlation analysis. Similarly to the findings in young animals, a positive significant correlation was observed between mRNA expression levels of Cpt1a in PBMCs and its expression levels in rWAT and liver at the age of 6 months. Moreover, a positive significant correlation was observed between expression levels of Rxrb in PBMCs and rWAT. No associations were found between mRNA levels of Fasn and Star in PBMCs and their expression levels in WAT or liver (data not shown).

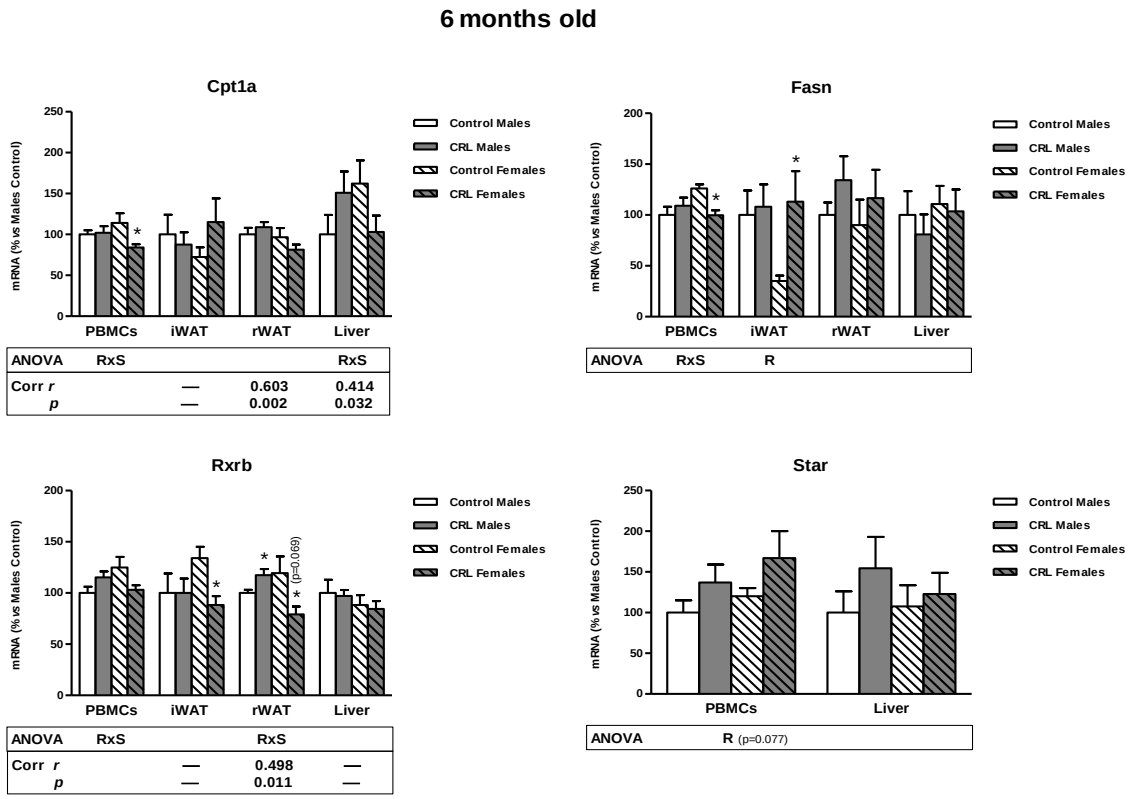

Figure 4. Comparison of mRNA expression levels of some lipid metabolism-related genes in PBMCs with the expression determined in inguinal and retroperitoneal WAT and liver of male and female offspring of control and at 6 months of age. Only those genes with significant differences between control and CRL animals in PBMCs of adult animals are shown. mRNA levels were measured by RT-qPCR, and expressed as a percentage of the mean value of males control. Data are mean ± S.E.M (n=6-8 animals/group). Statistics: R, effect of maternal calorie-restriction during lactation; RxS, an interaction between the effect of maternal calorie-restriction during lactation and the effect of sex ($p<0.05$; two-way ANOVA). *, CRL *vs* Controls ($p<0.05$; Student's *t* test); Corr, consistent positive correlations between mRNA expression levels of selected genes involved in lipid metabolism in PBMCs and mRNA expression levels of those genes in iWAT, rWAT and liver of male and female offspring of control and calorie-restricted dams during lactation (CRL) at the age of 6 months; *r*, Pearson's correlation coefficient; *p*-value of the genetic correlation ($p<0.05$); – indicates no correlations.

Discussion

Biomarkers derived from disease processes, such as those identified in obese subjects or in animal models of obesity, might not serve to assess the potential benefits of bioactive compounds or changes in dietary habits aiming to decrease the risk of obesity and related metabolic alterations in healthy or at-risk individuals. Therefore, in this study we took up this issue and aimed at identifying early transcript-related biomarkers of improved metabolic health, using an animal model programmed for reduced risk for obesity development and related metabolic alterations in adulthood. For reasons of accessibility and minimal invasiveness, a blood-derived RNA source, PBMCs, was used. Findings from this study reveal that transcript levels of lipid metabolism-related genes in PBMCs can be used as early biomarkers of metabolic health and potentially reflecting metabolic processes occurring in other tissues.

Early postnatal intervention based on moderate maternal calorie-restriction during lactation brought about improvements in some phenotypic traits in the offspring, such as body weight and fat content, as previously described (Palou et al. 2011). The effect of lowering body weight was observed in both males and females from the fifth day of life and was persistent when animals were adult, although it was more pronounced in females than in males. At weaning, both males and females also displayed lower body fat content, as well as lower weight of fat depots, but differences were only maintained in later life in females. Despite changes in body weight, body length of adult animals was not affected. The decrease of body weight of offspring could be partly due to lower food intake. CRL animals ate fewer calories than their controls, with the differences again more pronounced in females.

Circulating hormones, such as insulin and leptin, could also contribute to characterize the metabolic health of these animals in relation to a better ability to maintain energy homeostasis throughout life. Leptin and insulin are hormones related to central control of feeding behavior and energy expenditure (Schwartz et al. 2000). High leptin levels are associated with insulin resistance and metabolic syndrome (Esteghamati et al. 2009), whereas lower leptin levels have been associated with improvement of insulin sensitivity (Sanchez et al. 2008). At weaning, CRL animals showed lower plasma levels of insulin and leptin. This healthier profile of circulating hormones was sustained in adulthood, particularly in females.

Regarding gene expression, taking both males and females into account, 278 genes were differentially expressed between control and CRL animals in PBMCs at the age of 21 days. As PBMCs are a subset of white blood cells, it is not surprising that immune system was identified as the most affected process in terms of gene expression. This was followed by other processes such as signaling, cell turnover, transcription machinery, etc. Interestingly, also the expression of genes involved in metabolism of proteins, carbohydrates as well as lipids, and redox and central metabolism were affected. In a previous study using a similar animal model of 30% calorie-restricted dams during lactation, the offspring showed an improved capacity to handle and store excess dietary fuel in adulthood (Palou et al. 2011). We therefore focused on genes related to lipid metabolism.

Fatty acid synthase (FASN) catalyzes the formation of long-chain fatty acids from acetyl-CoA, malonyl-CoA and NADPH. Some studies have evidenced that the expression of Fasn in PBMCs reflects metabolic adaptations of the organism to fasting/feeding conditions. Fasn expression levels were found to be decreased with fasting and increased with refeeding in normoweight rats, however, this response was impaired in obese rats (Caimari et al. 2010a; Oliver et al. 2013). Here, it is shown that weaned CRL pups displayed lower Fasn mRNA levels than controls in PBMCs, and this decrease was also found in both WAT depots analyzed. This expression pattern was persistent in PBMCs at the age of 6 months, but only in females, in accordance with their higher protection (compared with males) against fat accumulation. Therefore, lower mRNA levels of this gene might be proposed as a marker for a higher protection against fat accumulation in adulthood, in accordance with the metabolic role of FASN in lipid synthesis.

The hormone-sensitive lipase (LIPE, better known as HSL), encoded by the Lipe gene, is an enzyme that hydrolyzes acylglycerols, as well as several other lipids stored in adipose tissue (Yeaman 2004). Although transcription of Lipe was initially described as specific for adipocytes, subsequent studies have shown that it is expressed and has a role in lipid metabolism in multiple tissues, including liver and macrophages (Yeaman 2004). Its action has also been suggested to be linked with insulin secretion and insulin action (Kraemer and Shen 2002). Expression of this gene has also been described in PBMCs, where its level was found to be reduced in “at risk” obese subjects (Telle-Hansen et al. 2013). Interestingly, in the present study we show that young CRL

animals exhibited higher Lipe mRNA levels in PBMCs and similarly in iWAT. Therefore, higher mRNA levels of this gene might be proposed as an early marker for a better capacity to regulate lipid metabolism in adulthood, possibly in association with improved insulin sensitivity. However, early changes were not persistent into adulthood, suggesting their potential usefulness as a biomarker only at early ages.

Cpt1a codes for carnitine palmitoyltransferase 1alpha (liver form). It mediates the transport of long-chain fatty-acids across the mitochondrial inner membrane and is rate limiting for their beta-oxidation. High expression levels of Cpt1a in PBMCs have been described in diet-induced obese rats (Caimari et al. 2010b). Similarly, Cpt1a mRNA levels in whole blood cells were higher in those overweight male children that seemed to be protected against the increase in plasma triglyceride levels associated with body fat accumulation (Sanchez et al. 2012). Conversely, lower expression of this gene in overweight male children was associated with higher HOMA index (Sanchez et al. 2012). In the present study, young CRL animals showed higher mRNA levels of Cpt1a in PBMCs than controls. Moreover, this expression pattern was correlated with that occurring in liver and WAT (both inguinal and retroperitoneal depots). However, differences found at early ages were not sustained in adulthood, neither in PBMCs nor in the tissues that were investigated, although the expression profile of this gene in PBMCs of adult animals was also related to the expression in rWAT and liver. Altogether, these results suggest positive metabolic effects related with high expression levels of Cpt1a in PBMCs at early ages in relation to increased oxidative capacity.

Rxrb encodes a member of the retinoid X receptor (RXR) family of nuclear receptors, which is involved in mediating the effects of 9-cis-retinoic acid. This protein has been linked with lipid metabolism, with dual effects. On the one hand, RXRb heterodimerizes with peroxisome proliferator-activated receptor alpha (PPARα), and in this way cooperates in the induction of the acyl-CoA oxidase gene, which encodes the rate-limiting enzyme of peroxisomal β-oxidation of fatty acids (Keller et al. 1993). On the other hand, RXR may also induce FASN via formation of LXR/RXR heterodimers binding to their recognition sequences in the sterol regulatory element-binding protein (SREBP-1c) promoter (Roder et al. 2007). The correlation between expression levels of this gene in PBMCs and rWAT in adulthood may be associated with the lower degree of adipogenesis that is observed.

The Lrp1 gene encodes the low density lipoprotein receptor-related protein 1 (LRP1), an endocytic receptor which is ubiquitously expressed in a variety of organs, including WAT, liver and brain, and is involved in several cellular processes (Hussain et al. 1999). Notably, Lrp1 has been described to be up-regulated in human and mouse obese adipose tissue, and silencing of Lrp1 expression in 3T3F442A murine preadipocytes brought about reduction of cellular lipid level that was associated with an inhibition of adipogenesis (Olivier et al. 2009). These observations suggest that changes in Lrp1 expression may have important consequences for fat accumulation and obesity, which agrees with the protection against fat accumulation in CRL animals.

Other genes, whose expression was changed in CRL *versus* control animals, include Pcyt2 and Star, which were down-regulated and up-regulated, respectively. Pcyt2 encodes CPT:phosphoethanolamine cytidylyltransferase, the main regulatory enzyme in the *de novo* ethanolamine phospholipids synthesis (Pavlovic and Bakovic 2013). The meaning of underexpression of this gene in PBMCs at an early stage of life, as occurring in CRL animals, remains to be determined. Nonetheless, it must be mentioned that microarrays results of this gene were confirmed by RT-qPCR only in females; a similar decrease was also found in WAT and liver of CRL females, but not males, and these changes were not maintained in adult rats. Hence, its function as a potential biomarker appears more limited. The Star gene encodes for the steroidogenic acute regulatory protein (STAR), which is a transport protein that modulates cholesterol transfer within mitochondria for the production of adrenal and gonadal steroids in steroidogenic tissues, representing the limiting step (Lin et al. 1995). This gene has also been described to be expressed in monocytes, macrophages, and human aortic tissue (Taylor et al. 2010). In macrophages, Star overexpression impacts positively on the lipid-related phenotype of these cells, since it represses a number of genes involved in cholesterol biosynthesis and LDL uptake, and markedly increases the expression of ABCA1, strongly suggesting that Star increases sterol efflux to apoAI (Taylor et al. 2010). This protein has also been shown to be a protective molecule for endothelial dysfunctions in aortic endothelium (Tian et al. 2012). Interestingly, presence of STAR protein has also been described in human liver cells, where it appears to be involved in cellular cholesterol homeostasis, representing a potential therapeutic target in the management of hyperlipidemia (Hall et al. 2005). Therefore, results from this study suggest that increased transcript levels of Star in blood cells of young CRL animals,

also reflecting changes occurring in liver, may be indicative of improved cholesterol metabolism and hence improved cardiovascular health. Notably, a trend to higher transcript levels of Star in PBMCs was also shown in CRL adult animals. Hence, it would be interesting to assess the relationship between PBMC transcript levels of Star and the ability to maintain normal blood cholesterol levels under appropriate dietary insults or in an animal model prone to develop hypercholesterolemia, since in the conditions of this study, no differences were found between groups concerning blood cholesterol (data not shown).

All in all, despite the fact that nutritional-induced changes in PBMC gene expression are generally expected to be smaller than the effects induced by diseases (Bouwens et al. 2007), early programming effects occurring in CRL animals due to calorie-restriction during lactation were reflected in a substantial number of changes. Among genes related with lipid metabolism, it is noteworthy that expression levels of Cpt1a, Fasn and Star in PBMCs at early ages were significantly correlated with expression profiles in WAT and/or liver.

To summarize, the findings from this study, using an animal model that confers certain protection in the pups against development of obesity and related metabolic alterations by dietary intervention in lactating dams, reveal the possibility of using transcript levels of lipid metabolism-related genes in PBMCs, particularly Fasn, Cpt1a and Star, as early biomarkers of metabolic health, potentially providing a valid biological readout for the study of metabolic processes in humans.

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IV. RECAPITULATION

RECAPITULATION

The results obtained during the development of this PhD thesis and presented in the manuscripts, bring novel data about the effects of early life nutritional conditions and factors, such as submission of pregnant or lactating dams to moderate maternal calorie restriction of 20% and neonatal leptin treatment, on the health outcomes in relation to obesity and related metabolic alterations. The particular effects of these early life events observed at morphological/histological and transcriptional level in metabolically relevant tissues, may help to elucidate the underlying mechanisms of metabolic programming. Notably, the use of these animal models of intervention in early life has enabled to identify a number of potential early biomarkers of metabolic health and dysfunction, by transcriptome profiling of PBMCs, an easily accessible surrogate tissue.

A poor prenatal nutrition programs an impaired ability to regulate energy homeostasis, and hence increases susceptibility to develop obesity and its related pathologies in adulthood. In this sense, the results obtained here show that moderate maternal calorie restriction of 20% during the first part of gestation in rats programs the offspring for insulin and leptin resistance (**manuscript 1**). The lower capacity to respond to central and peripheral action of insulin and to central leptin action is already present at early ages. In comparison to the controls, offspring of calorie-restricted dams during pregnancy (CR) exhibited lower mRNA expression levels of insulin receptor in the hypothalamus, WAT and liver, and long-form leptin receptor (Obrb) in hypothalamus at the age of 25 days. These alterations may explain the hyperphagia that showed these animals in adulthood (both genders), although only males developed higher body weight and fat content that their corresponding controls. In adulthood, CR animals also showed gender-dependent changes in the expression profile of key genes involved in the control of energy homeostasis. Specifically, at the age of 6 months, CR rats exhibited lower Obrb mRNA levels in the hypothalamus (only females, but both showed altered Npy/Pomc mRNA ratio), rWAT, and liver (males). CR animals also exhibited decreased levels of proteins involved in insulin and leptin signaling, such as protein kinase C zeta in WAT (females) and liver (males), and of phosphorylated STAT3 in liver (females). The expression levels of insulin receptor in peripheral tissues (WAT and liver) at early stages of life, together with the impaired action of insulin and leptin found in adult life, suggest that early programming of peripheral insulin resistance may be a direct consequence of fetal

caloric restriction, while peripheral leptin resistance, which appears in adulthood and under HF diet, might be secondary to insulin resistance or to central leptin resistance.

In addition to the overall detrimental effects of gestational calorie restriction on the health outcomes in the offspring, our results show that this prenatal condition impairs the thermogenic capacity of BAT (**manuscript 2**). Both male and female offspring of calorie restricted dams during pregnancy (CR) displayed a significant reduction of specific UCP1 protein levels in BAT compared to their controls. In agreement with the relationship between UCP1 production and sympathetic innervation in BAT, the reduced thermogenic capacity in the offspring could be due to the reduced sympathetic drive into tissue observed in CR animals. Thyroid hormones, which interact with the SNS, are needed for the full thermogenesis activation in BAT. In this sense, CR animals, and particularly males, presented decreased plasma T3 levels, together with lower expression levels of Dio2 in BAT, which encodes for the enzyme that locally produces T3, via 5' deiodination of thyroxine (T4). Therefore, both impaired sympathetic drive and altered thyroid hormone signaling could contribute to the reduced BAT burning capacity in CR animals. These alterations may account for the altered metabolic profile in BAT of CR animals, where a lower capacity for fatty acid uptake accompanies a limited fatty acid oxidation capacity, as mRNA expression levels of both Lpl and Cpt1 were downregulated. Moreover, the reduced BAT activity in CR animals impairs their capacity to maintain body temperature. In fact, when animals were exposed to cold, both male and female CR animals experienced a transient decline in body temperature during the first hours, although normal values were reestablished after 24-h. The recovery of normal body temperature might be attributed, at least in part, to a greater activation of shivering thermogenesis in skeletal muscle, as UCP1 production and body weight gain in CR animals during the period of cold exposure were lower than in controls. All in all, these findings suggest that disturbed burning capacity of BAT may contribute to the greater sensitivity to cold and greater predisposition to fat accumulation and other metabolic alterations that the offspring of calorie-restricted dams, and particularly males, suffer in adulthood.

Moderate calorie restriction during pregnancy in rats has also been previously associated with malprogramming of central hypothalamic structures involved in energy balance (Garcia et al. 2010), although the mechanisms or factors responsible are not clearly established. In rodents, leptin has been shown to play a crucial neurotrophic role in programming hypothalamic circuit formation (Bouret and Simerly 2006; Ahima et al. 1998). Neurodevelopment action of

leptin appears to be restricted to the second week of life, which is coincident with a rise in circulating leptin levels, the so-called leptin surge. In this sense, results from the present thesis have evidenced the absence of leptin surge in CR animals **(manuscript 1)**, which might be closely associated with the adverse health effects observed in these animals. Leptin is naturally present in maternal milk, and this exogenous leptin may substantially contribute to circulating leptin in neonate rats (Palou and Pico 2009). Notably, previous results obtained in our laboratory have shown that supplementation with physiological doses of leptin during lactation to neonate rats born from adequately nourished dams may prevent overweight in adulthood (Pico et al. 2007), as well as other alterations related with the metabolic syndrome (Priego et al. 2010). Regarding these issues, the results obtained here bring forward proof of the ability of oral leptin treatment throughout lactation to revert developmental malprogrammed effects exerted by moderate maternal calorie restriction during gestation on the offspring hypothalamic structure and function (**manuscript 3**). Specifically, leptin treatment during the suckling period normalized the total number of cells and cell density in ARC and PVN hypothalamic nuclei, and particularly restored decreased NPY^+ cells in ARC of CR males. According to the neurotrophic action of leptin, the restoration of the ARC structure could be a consequence, at least in males, of restored plasma leptin levels in these animals. These structural adjustments made by neonatal leptin treatment were accompanied by amelioration of hypothalamic mRNA expression levels of neuropeptides and factors involved in the regulation of feeding behavior. In CR male pups leptin treatment partially restored the decreased Npy, Cart and Socs-3 mRNA levels, and reverted the reduced Socs-3 mRNA levels found in CR females. In addition, the results show that leptin-treated male animals developed better responsiveness to leptin action, as they manifested higher mRNA expression levels of Obrb than control and CR animals. In turn, higher expression levels of Pomc in leptin-treated females with respect to their controls, may make them more predisposed for effective ways to control food intake in adulthood. These findings support evidence for the possibility to revert programming effects of an adverse prenatal environment, which has generally been considered an irreversible change in developmental trajectory, and for the importance of leptin intake in adequate amounts during neonatal period.

As previously shown by our laboratory group, moderate maternal calorie restriction during gestation not only affect hypothalamus structure in the offspring, but also exerts detrimental effects on WAT sympathetic innervation (Garcia et al. 2011). The results obtained here

revealed that the adverse programming effects of this prenatal condition on WAT structure and function may also be ameliorated by neonatal leptin supplementation (**manuscript 4**). Specifically, leptin treatment restored sympathetic drive into tissue (as evidenced by normalization of both TyrOH protein levels and its immunoreactive area), which was reduced in CR male animals but not in females. Moreover, leptin treatment brought about restoration of thyroid hormone (T3) plasma levels in male and female offspring, and mRNA expression levels of Dio2 in WAT of female animals. As SNS and thyroid hormones control adipocytes lipid metabolism, their restoration in leptin-treated male animals may account for normalization of impaired metabolic profile in WAT of CR male animals, as manifested by partial restoration of mRNA expression levels of genes involved in fatty acid uptake (Lpl) and catabolism (Atgl, Lipe, Cpt1b, Pgc1a). In turn, restoration of thyroid hormone signalling in leptin-treated females could be responsible for normalization of decreased Lpl mRNA levels in CR female animals. These adjustments induced by leptin treatment during lactation may result in a better handling and partitioning of excess fuel, and thus prevent the programmed predisposition to weight gain, fat accumulation and other metabolic abnormalities that CR animals, particularly males, undergo in adult life. This is another demonstration that a specific compound during lactation may reverse a detrimental trend for obesity induced by poor nutrition during pregnancy.

Beneficial effects of neonatal leptin treatment are not only restricted to structural corrections in solid tissues. The results evidenced that leptin supplementation is also able to normalize blood cells transcript-based potential early biomarkers associated to gestational calorie restriction (**manuscript 5**). Whole-genome transcriptome profiling of PBMCs from young (25-day-old) male offspring revealed that gestational food restriction affected significantly mRNA levels of 224 genes. Induced alterations in gene expression levels reflect a wide system of biological processes, as the changes were related to transcription and translation machinery, followed by genes related to immune system, signalling, cell turnover, metabolism, transport, cytoskeleton, sensory perception, nervous system and neural signaling. In turn, leptin treatment in CR males normalized the expression levels of almost of these genes (218) to control levels with the exception of 6 genes. Among the 218 genes whose expression levels were normalized, 196 of them were partially reverted and 22 were totally reverted. The latter 22 genes were selected for confirmation by qPCR. Of these 22 genes, qPCR analyses in PBMCs from males and females revealed that expression levels of several of them (Crmp1, Gla, Gls, Lrp11, Paox, Tmsb4x and Ubash3b) emerged as particularly

promising, because they expression levels were found to be quantifiable by qPCR and increased expression levels found in CR pups were totally reverted by leptin treatment in both genders. Although the exact function of each of these genes in PBMCs remains to be determined, these novel markers may allow early recognition and subsequent monitoring of individuals at early ages who are at higher risk to develop obesity and other pathologies and whose alterations can be reverted by the intake of breast milk with appropriate amounts of leptin during lactation. These results have contributed to the development of the patent entitled "Method for prediction and/or prevention of overweight, obesity and/or its complications through gene expression analysis".

While moderate maternal calorie restriction of 20% during pregnancy in rats has been associated with adverse health outcomes in adult offspring, prior results form our group showed that 30% calorie restriction during lactation protects the offspring from obesity development in adult life and from related metabolic alterations (Palou et al. 2010b; Palou et al. 2011b). The results obtained during this thesis also evidenced that a modest maternal calorie restriction of 20% during lactation programs the offspring for better metabolic health in terms of body weight and lipid handling (**manuscript 6**). The beneficial effects of this condition on the offspring metabolic health were more evident in females, and particularly when animals were exposed to HF diet feeding. In concrete, offspring of 20% calorie-restricted dams during lactation (CRL) showed lower body weight, as well as lower adiposity under HF diet conditions, with the differences being more evident in females. CRL animals ate fewer calories both under NF and HF diets, with the differences being also more marked in females. CRL animals also displayed a better profile of circulating parameters. Decreased levels of insulin, leptin and increased of adiponectin in CRL animals at early age, as well as decreased values of leptin/adiponectin ratio and HOMA index (particularly in females) in adulthood, suggest that these animals are programmed for improved insulin and leptin sensitivity. The protective effects of calorie restriction during lactation against dyslipidemia and hepatic steatosis were also evident, since both male and female adult CRL animals showed lower TG levels than controls under HF dietary stressor, and lower hepatic lipid content both under NF and HF diets. These beneficial effects observed in adult offspring may be attributed to the early adaptations that develop young CRL animals at transcriptional level in key tissues involved in energy homeostasis. Underexpression of lipogenesis-related genes together with overexpression of genes related to lipolysis and fatty acid oxidation in WAT and liver, suggests that CRL animals are programmed for better lipid handling. These animals

are also programmed for increased sensitivity to the peripheral effects of insulin and leptin, as evidenced by upregulation of genes involved in insulin (WAT) and leptin (liver) signaling. Some of these adaptations occurring in early ages are partially maintained in adulthood and are particularly evident when animals are exposed to an obesogenic environment. Among the genes that exhibit changes at the expression level in early ages, transcript levels of Irs1 in WAT and Obrb and Srebp1c in the liver in adult animals were associated with hepatic lipid content, circulating parameters (triglycerides and insulin) and insulin resistance indexes. These associations pinpoint the potential relevance of transcript levels of these genes as markers of metabolic health.

Furthermore, microarray analysis of PBMCs from control and the offspring of calorie-restricted dams during lactation allowed the identification of 278 genes differentially expressed between both groups of animals at the age of 21 days (**manuscript 7**). As PBMCs are a subset of white blood cells, immune system was identified as the most affected process in terms of gene expression, followed by signaling, cell turnover, and transcription machinery. Other processes with a notable number of genes were related to metabolism of proteins, carbohydrates and lipids. Among genes related to lipid metabolism, expression of Cpt1a, Lipe and Star was increased and Fasn, Lrp1 and Rxrb decreased in CRL versus controls, with changes fully confirmed by qPCR. These genes may be useful as potential biomarkers at early ages, as the expression profiles of most of them in PBMCs were not persistent into adulthood. Notably, among the aforementioned genes, Cpt1a, Fasn and Star emerged as particularly interesting, as their expression profiles reflected the changes occurring in key tissues involved in lipid metabolism. In this regard, transcript levels of Cpt1a in PBMCs correlated with their levels in WAT and liver at both ages examined; Fasn expression levels in PBMCs at an early age correlated with their expression levels in WAT; and early changes in Star expression levels in PBMCs correlated with their expression levels in liver and were sustained in adulthood. These findings reveal the possibility of using transcript levels of these genes in PBMCs as early biomarkers of metabolic health status, potentially providing a valid biological readout for the study of metabolic processes in humans.

All in all, the results obtained along this thesis have enable to identify a number of easily accessible early biomarkers reflecting metabolic health and dysfunction, what brings interesting expectations for future investigations. In this regard, validation of these biomarkers in other animal models with improved/impaired metabolic health as well as in

humans becomes of interest, as they might serve in the future as a basis to substantiate the effects of foods and food components on health and to implement personalized recommendations and nutritional interventions before the disease is manifested.

V. CONCLUSIONS/CONCLUSIONES

CONCLUSIONS

1. Moderate (20%) maternal calorie restriction during the first part of gestation in rats impairs insulin and central leptin signaling in the offspring, which may explain the hyperphagia and metabolic alterations that show these animals in adulthood. These effects may also be responsible for the increased body weight found in male animals in adulthood. Female animals appear to be more resistant to the detrimental effects of gestational calorie restriction, in terms of maintenance of body weight, in spite of the altered profile of gene expression in key tissues involved in energy homeostasis.

2. The aforementioned maternal condition during gestation also programs offspring for lower brown adipose tissue thermogenic capacity, which may be explained by impaired sympathetic innervation and thyroid hormone signalling in this tissue. These abnormalities occurring at the juvenile age may determine the greater sensitivity to cold that show these animals and contribute to the higher propensity for fat accumulation and other metabolic alterations later in life.

3. Adverse effects of moderate maternal calorie restriction during pregnancy on the offspring health could be associated with the absence of a transient increase in plasma leptin levels during the suckling period, the so-called "neonatal leptin surge".

4. Leptin supplementation throughout lactation is able to revert, at least partly, most of the developmental effects on hypothalamic and WAT structure and function caused by moderate maternal calorie restriction during gestation. Normalization of altered cellularity in ARC and PVN hypothalamic nuclei, and particularly the number of NPY cells in ARC nucleus was associated with amelioration of mRNA expression levels of neuropeptides and factors involved in the regulation of feeding behavior (Npy, Cart and Socs-3). In turn restoration of sympathetic innervation in WAT, together with normalization of reduced thyroid hormone plasma levels and its signaling in the adipose tissue, were also accompanied by partial normalization of mRNA expression levels of genes involved in fatty acid uptake (Lpl) and catabolism (Atgl, Lipe, Cpt1b, Pgc1a). These findings bring evidence supporting the relevance of the intake of leptin during lactation, a specific compound of breast milk with beneficial health effects, which might be worth considering when searching for strategies to treat and/or prevent programmed trend to obesity caused by inadequate fetal nutrition.

5. Whole-genome transciptomic analysis of PBMCs revealed that leptin supplementation throughout lactation to the offspring of calorie-restricted rats during gestation normalizes expression levels of most genes affected by this condition during pregnancy. Out of the 224 genes whose expression in PBMCs was altered due to maternal undernutrition, leptin treatment normalized the expression of 218 of them to control levels. These biomarkers may be useful for early identification and subsequent monitoring of individuals who are at risk of later diseases and would specifically benefit from the intake of appropriate amounts of leptin during lactation.

6. Maternal calorie restriction of 20% during lactation in rats programs the offspring for better metabolic health in terms of body weight and lipid handling. This condition determines early adaptations in WAT and liver, affecting lipogenic and oxidative capacity, and increasing their sensitivity to the peripheral effects of leptin and insulin, which suggests a better control of energy metabolism. These adaptations occurring in early ages are partially maintained in adulthood but are particularly evident when animals are exposed to an obesogenic environment. Among the genes that exhibit changes at the expression level in early ages, Irs1 (in WAT) and Obrb and Srebp1c (in the liver) are of relevance because their transcript levels in adult animals were associated with hepatic lipid content, circulating parameters such as triglycerides and insulin, and insulin resistance indexes; thus they could be considered as potential biomarkers of metabolic health status in adult animals.

7. Transcriptome profiling in PBMCs from control and the offspring of rats submitted to moderate calorie restriction during lactation allowed the identification of 278 genes differentially expressed between both groups at an early age. Transcript levels of these genes could be considered as potential early biomarkers of metabolic health. Among lipid metabolism-related genes, Cpt1a, Fasn and Star emerged as particularly interesting, as their expression levels in PBMCs at early ages were significantly correlated with expression profiles in WAT and/or liver, and may potentially provide a valid biological readout for the study of metabolic processes in humans.

CONCLUSIONES

1. Una restricción calórica moderada (20%) durante la primera parte de la gestación en ratas altera la sensibilidad a la insulina y la acción central de la leptina en la descendencia, lo cual puede explicar la hiperfagia y las alteraciones metabólicas que muestran estos animales en edad adulta. Estos efectos también pueden ser responsables del sobrepeso que padecen los animales machos en edad adulta. Las hembras parecen ser más resistentes a los efectos perjudiciales de una restricción calórica gestacional, en términos de mantenimiento de peso corporal, a pesar de mostrar un perfil de expresión génica alterado en tejidos clave implicados en la homeostasis energética.

2. Dicha condición en las madres durante la gestación también programa a la descendencia para una menor capacidad termogénica en el tejido adiposo marrón, que puede explicarse por una deficiente inervación simpática y una alterada señalización de la hormona tiroidea en este tejido. Estas anormalidades que suceden en la edad juvenil pueden determinar la mayor sensibilidad al frio que presentan estos animales y contribuir a la mayor propensión a la acumulación de grasa y otras alteraciones metabólicas en edades más avanzadas.

3. Los efectos adversos de una restricción calórica moderada durante el embarazo sobre la salud de la descendencia podrían asociarse a la ausencia de un incremento transitorio en los niveles plasmáticos de leptina durante el periodo de lactancia, conocido como "pico de leptina neonatal".

4. La suplementación con leptina durante la lactancia es capaz de revertir, al menos en parte, la mayoría de los efectos sobre el desarrollo y función del hipotálamo y del tejido adiposo blanco (TAB) causados por una restricción calórica moderada durante la gestación. La normalización de la alterada celularidad en los núcleos hipotalámicos ARC y PVN, y particularmente del número de células NPY en el núcleo ARC, se asoció con una mejora de la expresión de neuropéptidos y factores involucrados en la regulación de la conducta alimentaria (Npy, Cart y Socs-3). A su vez, el restablecimiento de la inervación simpática en el TAB, junto con la normalización de la concentración plasmática de la hormona tiroidea y su señalización en el tejido adiposo, también se acompañó de la normalización parcial de los niveles de ARNm de genes involucrados en la captación de ácidos grasos (Lpl) y su catabolismo (Atgl, Lipe, Cpt1b, Pgcla). Estos hallazgos evidencian la

relevancia de la ingesta de leptina durante la lactancia, un compuesto específico de la leche materna con efectos beneficiosos para la salud, que merece ser considerado en la búsqueda de estrategias para tratar y/o prevenir una mayor tendencia a la obesidad causada por una inadecuada nutrición fetal.

5. El análisis transcriptómico de las PBMCs reveló que la suplementación con leptina durante la lactancia a las crías de ratas sometidas a restricción calórica durante la gestación normaliza los niveles de expresión de la mayoría de genes afectados por dicha condición durante el embarazo. De los 224 genes, cuya expresión en PBMCs se vio alterada debido a una desnutrición materna, el tratamiento con leptina normalizó la expresión de 218 a valores controles. Estos biomarcadores pueden ser útiles para la identificación temprana y subsiguiente seguimiento de individuos que están en riesgo padecer futuras enfermedades y que podrían beneficiarse especialmente de la ingesta de cantidades apropiadas de leptina durante la lactancia.

6. La restricción calórica maternal del 20 % durante la lactancia en las ratas programa la descendencia para una mejor salud metabólica en términos de peso corporal y procesamiento de lípidos. Esta condición determina adaptaciones tempranas en el TAB e hígado, afectando la capacidad lipogénica y oxidativa, e incrementando su sensibilidad a los efectos periféricos de la leptina e insulina, lo cual sugiere un mejor control del metabolismo energético. Estas adaptaciones, que suceden en edades tempranas, son mantenidas parcialmente en la edad adulta pero son particularmente evidentes cuando los animales son expuestos a un entorno obesogénico. Entre los genes que mostraron cambios en su expresión en edades tempranas, son destacables Irs1 (en TAB), y Obrb y Srebp1c (en el hígado) porque la concentración de sus transcritos en edad adulta se asoció con el contenido hepático de lípidos, parámetros circulantes tales como triglicéridos e insulina, e índices de resistencia a la insulina; por lo tanto podrían ser considerados potenciales biomarcadores del estatus de salud metabólica en edad adulta.

7. El perfil transcriptómico en PBMCs de crías de ratas controles y de ratas sometidas a una restricción calórica durante la lactancia permitió la identificación de 278 genes expresados diferencialmente entre ambos grupos en una edad temprana. Los niveles transcriptómicos de estos genes podrían ser considerados como potenciales biomarcadores tempranos de salud metabólica. De los genes relacionados con el metabolismo lipídico, Cpt1a, Fasn y Star resultaron particularmente interesantes ya que sus

niveles de expresión en PBMCs en edades tempranas se correlacionaron significativamente con los perfiles de expresión en TAB y/o hígado, pudiendo proporcionar una potencial

VI. REFERENCES

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VII. ANNEX I. Patent

MINISTERIO DE INDUSTRIA, TURISMO Y COMERCIO

Oficina Española de Patentes y Marcas

Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica, utilizando la conexión segura de la O.E.P.M. Asimismo, se le ha asignado de forma automática un número de solicitud y una fecha de recepción, conforme al artículo 14.3 del Reglamento para la ejecución de la Ley 11/1986, de 20 de marzo, de Patentes. La fecha de presentación de la solicitud de acuerdo con el art. 22 de la Ley de Patentes, le será comunicada posteriormente.

Número de solicitud:	P201430428	
Fecha de recepción:	26 marzo 2014, 14:07 (CET)	
Oficina receptora:	OEPM Madrid	
Su referencia:	ES1597.19	
Solicitante:	UNIVERSITAT DE LES ILLES BALEARS	
Número de solicitantes:	2	
País:	ES	
Título:	MÉTODO PARA LA PREDICCIÓN Y/O LA PREVENCIÓN DE SOBREPESO, OBESIDAD Y/O SUS COMPLICACIONES MEDIANTE ANÁLISIS DE EXPRESIÓN GÉNICA	
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MINISTERIO
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(1) MODALIDAD: **PATENTE DE INVENCIÓN** **MODELO DE UTILIDAD**	[✓] [ ]
(2) TIPO DE SOLICITUD: PRIMERA PRESENTACIÓN ADICIÓN A LA PATENTE EUROPEA ADICIÓN A LA PATENTE ESPAÑOLA SOLICITUD DIVISIONAL CAMBIO DE MODALIDAD TRANSFORMACIÓN SOLICITUD PATENTE EUROPEA PCT: ENTRADA FASE NACIONAL	[✓] [ ] [ ] [ ] [ ] [ ] [ ]
(3) EXP. PRINCIPAL O DE ORIGEN: MODALIDAD: N.º SOLICITUD: FECHA SOLICITUD:	
4) LUGAR DE PRESENTACIÓN:	OEPM, Presentación Electrónica
(5-1) SOLICITANTE 1: DENOMINACIÓN SOCIAL: UNIVERSIDAD PÚBLICA NACIONALIDAD: CÓDIGO PAÍS: NIF/NIE/PASAPORTE: CNAE: PYME: DOMICILIO: LOCALIDAD: PROVINCIA: CÓDIGO POSTAL: PAÍS RESIDENCIA: CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: PERSONA DE CONTACTO: MODO DE OBTENCIÓN DEL DERECHO: INVENCIÓN LABORAL: CONTRATO: SUCESIÓN: PORCENTAJE DE TITULARIDAD:	UNIVERSITAT DE LES ILLES BALEARS [ ] España ES Q0718001A Carretera de Valldemossa, Km.7,5 Palma de Mallorca 07 Islas Baleares 07122 España ES [✓] [ ] [ ] 090,00 %
(5-2) SOLICITANTE 2: DENOMINACIÓN SOCIAL: UNIVERSIDAD PÚBLICA NACIONALIDAD: CÓDIGO PAÍS: NIF/NIE/PASAPORTE: CNAE: PYME: DOMICILIO: LOCALIDAD:	CENTRO DE INVESTIGACIÓN BIOMÉDICA EN RED FISIOPATOLOGÍA DE LA OBESIDAD Y NUTRICIÓN [ ] España ES G84884428 C/ Choupana, s/n - Hospital Clínico de Santiago, Edf. D Planta 1ª Santiago de Compostela

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CONTRATO:	[ ]
SUCESIÓN:	[ ]
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CÓDIGO PAÍS:	PL
NIF/NIE/PASAPORTE:	
(6-4) INVENTOR 4:	
APELLIDOS:	SÁNCHEZ ROIG
NOMBRE:	JUANA
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	
(6-5) INVENTOR 5:	
APELLIDOS:	PALOU MARCH
NOMBRE:	MARIONA
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	
(7) TÍTULO DE LA INVENCIÓN:	MÉTODO PARA LA PREDICCIÓN Y/O LA PREVENCIÓN DE SOBREPESO, OBESIDAD Y/O SUS COMPLICACIONES MEDIANTE ANÁLISIS DE EXPRESIÓN GÉNICA
(8) PETICIÓN DE INFORME SOBRE EL ESTADO DE LA TÉCNICA:	
SI	[ ]
NO	[✓]
(9) SOLICITA LA INCLUSIÓN EN EL PROCEDIMIENTO ACELERADO DE CONCESIÓN	
SI	[ ]
NO	[✓]
(10) EFECTUADO DEPOSITO DE MATERIA BIOLÓGICA:	
SI	[ ]
NO	[✓]

(11) DEPÓSITO: REFERENCIA DE IDENTIFICACIÓN: INSTITUCIÓN DE DEPOSITO: NÚMERO DE DEPOSITO: ACCESIBILIDAD RESTRINGIDA A UN EXPERTO (ART. 45.1. B):	
(12) DECLARACIONES RELATIVAS A LA LISTA DE SECUENCIAS: LA LISTA DE SECUENCIAS NO VA MÁS ALLÁ DEL CONTENIDO DE LA SOLICITUD LA LISTA DE SECUENCIAS EN FORMATO PDF Y ASCII SON IDENTICOS	 [ ] [ ]
(13) EXPOSICIONES OFICIALES: LUGAR: FECHA:	
(14) DECLARACIONES DE PRIORIDAD: PAÍS DE ORIGEN: CÓDIGO PAÍS: NÚMERO: FECHA:	
(15) AGENTE DE PROPIEDAD INDUSTRIAL: APELLIDOS: NOMBRE: CÓDIGO DE AGENTE: NÚMERO DE PODER:	 PONS ARIÑO ÁNGEL 0499/5
(16) RELACIÓN DE DOCUMENTOS QUE SE ACOMPAÑAN: DESCRIPCIÓN: REIVINDICACIONES: DIBUJOS: RESUMEN: FIGURA(S) A PUBLICAR CON EL RESUMEN: ARCHIVO DE PRECONVERSION: DOCUMENTO DE REPRESENTACIÓN: JUSTIFICANTE DE PAGO (1): LISTA DE SECUENCIAS PDF: ARCHIVO PARA LA BUSQUEDA DE LS: OTROS (Aparecerán detallados):	 [✓] N.º de páginas: 54 [✓] N.º de reivindicaciones: 97 [✓] N.º de dibujos: 1 [✓] N.º de páginas: 1 [ ] N.º de figura(s): [✓] [✓] N.º de páginas: 1 [✓] N.º de páginas: 1 [ ] N.º de páginas: [ ]
(17) EL SOLICITANTE SE ACOGE AL APLAZAMIENTO DE PAGO DE TASA PREVISTO EN EL ART. 162 DE LA LEY 11/1986 DE PATENTES, DECLARA: BAJO JURAMIENTO O PROMESA SER CIERTOS TODOS LOS DATOS QUE FIGURAN EN LA DOCUMENTACIÓN ADJUNTA: DOC COPIA DNI: DOC COPIA DECLARACIÓN DE CARENCIA DE MEDIOS: DOC COPIA CERTIFICACIÓN DE HABERES: DOC COPIA ÚLTIMA DECLARACIÓN DE LA RENTA: DOC COPIA LIBRO DE FAMILIA: DOC COPIA OTROS:	[ ] [ ] N.º de páginas: [ ] N.º de páginas: [ ] N.º de páginas: [ ] N.º de páginas: [ ] N.º de páginas: [ ] N.º de páginas:
(18) NOTAS:	
(19) FIRMA: FIRMA DEL SOLICITANTE O REPRESENTANTE: LUGAR DE FIRMA: FECHA DE FIRMA:	 ENTIDAD PONS PATENTES Y MARCAS INTERNACIONAL SL - CIF B84921709 - NOMBRE PONS ARIÑO ANGEL - NIF 50534279J MADRID 26 Marzo 2014

VIII. ANNEX II. Materials and methods

Annex II. Materials and methods

1. Experimental animals

The experiments were conducted on male and female Wistar animals purchased from Charles River Laboratories (Barcelona, Spain). During experimentation animals were kept in animal house facility located at the University of the Balearic Islands, under standard conditions: controlled temperature (22°C), the normal 12h light and 12h dark cycle, free access to tap water and a standard laboratory rodent chow diet (3 kcal/g, with 8% calories from fat; Panlab) (Barcelona, Spain) unless specify. Rats were sacrificed by decapitation and selected tissues were collected, rinsed with saline solution with 0.1% diethylpyrocarbonate (DEPC) (Sigma) to avoid RNA degradation, and stored at −80°C until analysis. The stomach samples were opened longitudinally and were carefully rinsed in saline solution with 0.1% DEPC. Then, the stomach samples were cut into two pieces and stored at −80°C until analysis (one part was destined for protein analysis and the other for gene expression analysis). Moreover, trunk blood samples were collected for plasma and peripheral blood mononuclear cells (PBMCs) isolation (described in the section 2). For plasma, blood samples were collected in heparinized tubes, centrifuged at 700*g* for 10 min and stored at -20ºC until analysis.

2. PBMCs isolation

PBMCs, a subset of white blood cells including lymphocytes and monocytes, were isolated from the samples of trunk blood by density gradient separation using two types of mediums, Ficoll-Paque™ PLUS (GE Healthcare) and OptiPrep™ Density Gradient Medium (Axis-Shield).

Density gradient centrifugation is a technique that allows the separation of cells, organelles and macromolecules, depending on their size, shape and density. A density gradient is created in a centrifuge tube by layering solutions of varying densities with the dense end at the bottom of the tube. Differential migration during centrifugation results in the formation of layers containing different cell types. When isolating PBMCs, the bottom layer contains erythrocytes which have been aggregated by the medium. The layer above the erythrocytes contains mostly granulocytes and the lymphocytes are found at the interface (buffy coat) between the plasma and the medium with other slowly sedimenting particles (platelets and monocytes).

Isolation of PBMCs with Ficoll density-gradient centrifugation assay

The gravity sedimentation in a shallow Ficoll-Paque™ PLUS (GE Healthcare) density gradient, which is Ficoll sodium diatrizoate solution of the proper density (1.077 g/ml), viscosity, and osmotic pressure for use in a simple and rapid lymphocyte isolation procedure, was performed according to the instructions of the manufacturer.

Blood samples were collected using heparin in sodium chloride (NaCl) 0.9% solution as anticoagulant (200 µl of heparin in NaCl 0.9% for 500-600 µl of blood), and then diluted with an equal volume of balanced salt solution (BSS) (200 µl of heparin in NaCl 0.9% + 500 µl of blood + 700 µl of BSS). Blood treated with anticoagulant was then layered on the Ficoll-Paque PLUS solution (1.5 ml of Ficoll for 2 ml of blood mixed with balanced salt solution and heparin in NaCl) and centrifuged at 900*g* for 40 min at 20°C (with minimum acceleration and deceleration). After centrifugation and layers formation, the cell ring fraction (buffy coat) containing white lymphocytes were harvested. This material was then washed by centrifugation in balanced salt solution (3 volumes of BSS) at 900*g* for 10 min at 20°C to remove any platelets, Ficoll-Paque PLUS and plasma, and finally frozen at -80°C until RNA extraction.

Reagents:

- Heparin 0.2% in NaCl 0.9%: 0.2 g of heparin (Sigma) in 100 ml of NaCl 0.9%
- Balanced Salt Solution (BSS): 1:9 mixture of solution A and solution B. Solution A: 5.5mM anhydrous D-glucose (Merck), 5mM $CaCl_2$ x $2H_20$ (Pancreac), 0.98mM $MgCl_2$ x $6H_20$ (Pancreac), 5.4mM KCl (Pancreac), 145mM Trisma base (Sigma), pH adjusted to 7.6. Solution B: 140mM NaCl.
- Ficoll-Paque™ PLUS (GE Healthcare)

Isolation of PBMCs with OptiPrep™ Density Gradient Medium

Isolation of PBMCs with OptiPrep™ (Axis-Shield) was performed according to the manufacturer's instructions. OptiPrep™ is a medium based on iodixanol, which with an adequate density (1.320 g/ml) and osmolality (170 mOsm) provide a reliable and simple method for the preparation of mononuclear cells (PBMCs) from rodent and rabbit blood, which have a higher density than those from human blood.

Blood samples were collected using 100mM EDTA (pH 8.0) as anticoagulant (the final concentration of EDTA after the addition of blood should be 4mM, e.g. 40 µl of 100mM

EDTA (pH 8.0) for 1 ml of blood was used). Then, the collected blood was diluted with Solution C to a he final volume 6 ml. Subsequently, 3 ml of Density barrier was dispensed in the new tube and 6 ml of diluted blood was layered carefully over the density barrier, and then centrifuged at 700*g* for 20 min at room temperature with minimum acceleration and deceleration. After centrifugation, the PBMCs formed a sharp band at the interface. Most of the plasma layer (rich in RNAses) was removed and the band containing PBMCs from the meniscus downwards was collected carefully, without taking the layer under the PBMC band, consisting of thrombocytes and erythrocytes. Then, the cell harvest was rinsed with 2 volumes of solution C (about 2-3 ml) to reduce the density of the solution. PBMCs were pellet by centrifugation at 400*g* for 10 min at room temperature with minimum acceleration and deceleration. The supernatant was removed completely and the pellet was then frozen at -80°C until RNA extraction.

Reagents:

- OptiPrep™ Density Gradient Medium (Invitrogen)
- 0.5M EDTA stock solution, pH 8.0: 186.1 g ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate (Sigma) dissolved in 800 ml of distilled water, 20 g of NaOH pellets (Pancreac), pH adjusted to 8.0 and filled up to 1 liter with distilled water
- 100mM EDTA working solution: 1 part of 0.5M EDTA stock solution diluted with 4 parts of distilled water
- Solution C: 0.85 g NaCl (Pancreac) dissolved in 80 ml distilled water, 1 ml 1M HEPES (Sigma), pH adjusted to 7.4 and filled up to 100 ml with distilled water
- OptiPrep diluent: 2.5 volumes of Solution C diluted with 0.5 volumes of distilled water (eg. 10 ml of Solution C mixed with 2.0 ml distilled water).
- Density barrier: 2.7 volumes of OptiPrep ™ Density Gradient Medium (Invitrogen) diluted with 9.3 volumes of OptiPrep diluent (e.g. 2.7 ml OptiPrep mixed with 9.3 ml OptiPrep diluents).

3. RNA extraction and quantification

Three different methods were used to extract RNA from tissues depending on the type of tissue and its size. In general, when the entire tissue needed to be homogenized for gene expression analysis (e.g. hypothalamus or stomach) or the size of tissue was small (particularly tissues collected from young animals), the extraction was performed with use of commercial TriPure Reagent (Roche Applied Science) or NucleoSpin® TriPrep kit

(Macherey-Nagel). These methods enabled also to extract protein and DNA at the same time of RNA extraction (TriPrep kit) or to obtain their phases that might be further processed if needed (TriPure). For relatively larger size of tissue or when the intended use of extracted yield required its high quality and purity (e.g. use of RNA from PBMCs for microarray analysis), RNA was extracted with use of E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek) or NucleoSpin® TriPrep kit (Macherey-Nagel). At any rate the extraction was realized according to the manufacturer´s guidelines.

Procedure I. Extraction of total RNA from samples using TriPure Reagent

The isoslation with TriPure Reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, allows maintaining the integrity of the RNA and denatures endogenous nuclease, while disrupting cells and dissolving cell components.

Tissue samples (whole hypothalamus and stomach samples or 100 mg of liver or adipose tissue) were homogenized precisely with 1 ml of TriPure Reagent. In case of adipose tissue and hypothalamus, centrifugation at 12000g for 10 min was applied to remove lipids content. To separate RNA, 200 µl of chloroform was added to the mixture, mixed vigorously for 15 s, incubated 5-15 min on ice, and then centrifuged at 12000*g* for 10 min at 4°C. In this way the sample was separated into three phases: a colorless aqueous (upper) phase with RNA that was transferred to a separate fresh tube, a white interphase and a red phenol-chloroform (lower) phase containing proteins and DNA and organic phase, which was stored at -20°C. RNA was precipitated by adding 500 µl of isopropanol, the samples were inverted several times and incubated at least 10 min at room temperature or kept at -20°C overnight. Subsequently, samples were centrifuged at 12000*g* for 15 min at 4°C and the supernatant (isopropanol) was discarded. The precipitate with RNA was then washed with 75% ethanol (1 ml of 75% ethanol per 1 ml of TriPure Reagent), and tubes were shacked vigorously. The samples were centrifugedat 7500*g* for 5 min at 4°C, the supernatant was discarded, and the RNA pellet was dried (air-dry) for 5-10 min to remove the any rests of ethanol. Finally, RNA-ase free water (20-200 µl) was added to each tube, mixed well and stored at -80°C.

Reagents:

- TriPure Reagent (Roche Applied Science)
- Chloroform (Sigma)
- Isopropanol (Sigma)
- Ethanol absolute (Pancreac)

- RNA-ase free water (Sigma)

Procedure II. Extraction of total RNA from samples using E.Z.N.A.® Total RNA commercial kit

The E.Z.N.A.® Total RNA Kit I provides a simple and rapid method to obtain purified RNA from relatively low amount of tissue (up to 1 x 10^7 eukaryotic cells or 30 mg). This system uses the reversible binding properties of the HiBind Matrix in combination with the speed of mini-column spin technology thereby permitting single or multiple simultaneous processing of samples. Samples are applied to the HiBind RNA spin column to which total RNA binds. Cellular debris and other contaminants are effectivelywashed away after a few quick wash steps.

Tissue samples (PBMCs, 10 mg of liver or 10-15 mg of adipose tissue) were homogenized in ice with 350 µl of TRK Lysis Buffer (700 µl for adipose tissue). Optionally 2-mercaptoethanol (ß-mercaptoethanol) was added to an aliquot of TRK Lysis Buffer before use (20 µl of 2-mercaptoethanol per 1 ml of TRK Lysis Buffer), to reduce RNA degradation. Samples were centrifugedat full speed (13000*g*) for 5 min at room temperature. Cleared supernatant was transferred carefully to fresh tubes, 350 µl (or 700 µl) of 70% ethanol was added to the lysate and the mixture was mixed thoroughly by pipetting up and down 3-5 times. The samples were transferred to a HiBind RNA spin column placed into a 2 ml collection tube, centrifugedat 10000g for 60 s at room temperature and flow-through was discarded. Then, samples were washed with 350 µl of RNA Wash Buffer I, centrifugedat 10000*g* for 60 s and the flow-through was discarded. To remove DNA completely, 75 µl of the DNase I stock solution (73.5 µl of DNase I Digestion Buffer and 1.5 µl of RNase Free DNase) for each HiBind RNA column was pipetted directly onto center of column matrix placed into a new 2 ml collection tube and the samples were incubated at room temperature for 15 min. After DNase digestion, samples were washed with 400 µl of RNA Wash Buffer I, and then with 500 µl and 350 µl of RNA Wash Buffer II, centrifuged at 10000*g* for 60 s and the flow-through was discarded. To completely dry the HiBind matrix, samples with the empty collection tubes were centrifuged for 2 min at maximum speed (20000*g*). Finally, in order to elute the RNA, DEPC-treated water (20-40 µl) was added directly onto the center of the column matrix, centrifuged for 2 min at 10000g and stored at -80°C.

Reagents:

- RNA isolation kit (E.Z.N.A.® Total RNA Kit I) (Omega Bio-Tek)

- Ethanol absolute (Pancreac)
- ß-mercaptoethanol (Sigma)

Procedure III. Extraction of total RNA from samples using NucleoSpin® TriPrep commercial kit

The NucleoSpin® TriPrep kit enables isolation of DNA, RNA, and protein from diverse sample types. DNA, RNA, and protein are isolated from one and the same sample, which is especially valuable for unique, small, and precious samples. DNA and RNA are eluted separately from the NucleoSpin® TriPrep Column, with a low salt buffer and water, respectively. One of the most important aspects in the isolation of DNA, RNA, and protein is to prevent their degradation during the isolation procedure. With the NucleoSpin® TriPrep method, cells are lysed by incubation in a solution containing large amounts of chaotropic ions. This lysis buffer immediately inactivates virtually all enzymes (e.g., DNases, RNases, proteases, and phosphatases) which are present in almost all biological materials.

Tissue samples (30 mg of white adipose tissue) were homogenized precisely with 350 µl of Buffer RP1 and 3.5 µl of ß-mercaptoethanol (per sample). In order to reduce viscosity and clear the lysate by filtration, the mixture was applied into the NucleoSpin® Filter equipped with a collection tube and centrifuge for 1 min at 11000*g*. The flow-through was transferred into a new 1.5 ml microcentrifuge tube, 350 µl of ethanol (70%) was added and the mixture was mixed by vortexing. The lysate was loaded into NucleoSpin® TriPrep Column placed in a collection tube and centrifuged for 30 s at 11000*g*. After centrifugation, RNA and DNA became bound to the column membrane, and the protein remained in the flow-through. The flow-through was recovered and stored at 4°C for further protein purification.

For DNA and RNA isolation, chaotropic salt was removed from the silica membrane by two-time washing of NucleoSpin® TriPrep Column with 500 µl of DNA Wash. Each washing was followed by the centrifugation for 1 min at 11000*g* and formed flow-through was discarded. Subsequently, the membrane of the NucleoSpin® TriPrep Column was dried for 3 min at benchtop, and then 100 µl of DNA Elute was added directly onto the membrane and incubate for 1 min. DNA was eluted by centrifugation for 1minat 11000*g* and stored at -20°C.

For RNA isolation, residual DNA on column was digested by adding 95 µl of rDNase reaction mixture (10 µl of reconstituted rDNase mixed with 90 µl of Reaction Buffer for rDNase, per sample) directly onto the center of the silica membrane of the column, followed by incubation at room temperature for 15 min. The rDNase was then inactivated by adding

200 µl of Buffer RA2 to the NucleoSpin® TriPrep Column and centrifugation for 30 s at 11000*g*. Silica membrane was then cleaned by two-time washing with 600 and 250 µl of Buffer RA3. Each washing was followed by the centrifugation at 11000g for 30 s and 2 min (to dry the membrane), respectively, and formed flow-through was discarded. Finally, the RNA was eluted in 40 µl of RNase-free water, centrifuged at 11000*g* for 1min and stored at -80°C.

For the protein precipitation, 500 µl of flow-through (the ethanolic lysate which has been passed through the NucleoSpin® TriPrep Column at the beginning of the procedure) was mixed with one volume of PP (Protein Precipitator), shacked vigorously and the mixture was incubated at room temperature for approximately 30 min. Supernatant was removed by pipetting, the pellet was washed with 500 µl of 50% ethanol and the mixture was centrifuged 1 min at 11000*g*. After removing the supernatant by pipetting, protein pellet was dried for 5–10 min at room temperature keeping the lid open. Protein sample was then prepared by adding 100 µl of PSB-TCEP (Protein Solving Buffer, containing reducing agent), followed by the incubation for 3 min at 95–98°C to completely dissolve and denature protein. In order to pellet residual insolvable material, centrifugation for 1 min at 11000*g* was applied and supernatant was recovered and stored at -80°C.

Reagents:

- RNA isolation kit (NucleoSpin® TriPrep kit) (Macherey-Nagel)
- Ethanol absolute (Pancreac)
- ß-mercaptoethanol (Sigma)

<u>RNA quantification and assessment of its integrity</u>

Determination of RNA quantity and quality was performed with use of the Nanodrop UV/Vis Spectrophotometer (NanoDrop ND-1000) and its integrity was assessed using 1.0% agarose gel electrophoresis.

Total RNA quantification: NanoDrop ND-1000 Spectrophotometer is used for quantification of nucleic acids usually using UV absorption. The concentration of nucleic acid can be determined using the Beer-Lambert law, which predicts a linear change in absorbance with concentration. An A260 reading of 1.0 is equivalent to about 40 µg/ml of RNA and the OD at 260 nm is used to determine the RNA concentration in a solution. RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the

RNA purity of an RNA preparation. Pure RNA has an A260/A280 of 1.8-2.0, in turn, the value superior than 2 determinate contamination with DNA, and minor than 1.8 – with proteins. In addition, spectrophotometric measurement informs about A260/230 ratio, which indicates the contamination degree with organic dissolvent (the proper value is ~2). For spectrophotometric quantification of isolated total RNA 2 µl of sample was used.

Verification of RNA integrity: Agarose gel electrophoresis technique relies on the negative charge of RNA/DNA for size separation in a sieving matrix. Visualization of 28S and 18S rRNA bands is an indicator of sample integrity. Intact total RNA is characterized by sharp bands, from which the 28S rRNA band should be approximately twice as intense as the 18S rRNA band. Completely degraded RNA appears as a very low molecular weight smear.

Total RNA (250 ng) was mixed with Loading Buffer and loaded into 1% agarose gel, prepared by dissolving agarose in Tris buffer solution and adding SYBR® Safe DNA gel stain (1 µl SYBR Safe for each 10 ml of 0.5 x Tris buffer), which is also suitable for staining RNA in gels. Gel was run at 80 V/cm until the blue indicator has migrated so far as 2.5 cm of the length of the gel. Bound to nucleic acids, SYBR® Safe stain has fluorescence excitation maxima at 280 and 502 nm, and an emission maximum at 530 nm. RNA bands stained with SYBR® Safe DNA gel stain were visualized using UV transilluminator (ChemiGenius) connected with a program GeneSnap. After exposition of the gel to UV light, the emitted fluorescence was photographed and sharpness of 28S and 18S rRNA bands was evaluated.

Reagents:

- RNA-ase free water (Sigma) or DEPC-treated water (Omega Bio-Tek) (to perform Blank measurement on NanoDrop Spectrophotometer the corresponding water was used, depending on the type of dissolvent used when isolating RNA)
- Agarose (Pronadisa)
- Electrophoresis running buffer: TBE 0.5X (Tris/Borate/EDTA: 44.5mM Tris base, 44.5mM Boric acid, 1mM EDTA)
- Loading buffer: 50% glicerol (Sigma), 0.25% Bromophenol blue (Panreac).
- SYBR Safe DNA gel stain (Invitrogen)

4. RT-qPCR analysis

Firstly, total cellular RNA was reverse transcribed into complementary DNA (cDNA), and then the resulting cDNA was used in quantitative polymerase chain reaction (RT-qPCR) to amplify and simultaneously quantify a targeted cDNA molecule.

Reverse transcription of total RNA to cDNA(RT)

Different protocols were assessed dependent on the mature of mRNA sample.

- RNA samples isolated from different depots of adipose tissue, hypothalamus, liver and stomach

Isolated total RNA (0.25 µg diluted in 5 µl of RNA-ase free water) was firstly denaturalized at 65°C for 10 min in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems). For RNA samples from different depots of adipose tissue, hypothalamus and liver, 7.5 µl of RT-mix per sample was taken to proceed with RT reaction. RT-mix consisted of: 1.25 µl of 10x buffer, 1.25 µl of 25mM $MgCl_2$, 2 µl of 2.5mM dNTPs, 0.5 µl of Random Hexamers, 0.5 µl of RNase Inhibitor, 0.5 µl of the enzyme Reverse Transcriptase and 1.5 µl of RNA-ase free water; and RT conditions were as follows: 15 min at 20°C, 30 min at 42°C and a final step of 5 min at 95°C. For RNA samples from stomach, 5 µl of RT-mix per sample was taken to proceed with RT reaction. RT-mix consisted of: 1 µl of 10x buffer, 1 µl of 25mM $MgCl_2$, 2 µl of 2.5mM dNTPs, 1 µl of Random Hexamers, 0.5 µl of RNase Inhibitor, 0.5 µl of Reverse Transcriptase; and RT conditions were as follows: 60 min at 42°C and 5 min at 95°C.

Reagents:

- RNA-ase free water (Sigma)
- RNase Inhibitor 20 U/µl (Applied Biosystems)
- Random Hexamers 50 U/µl (Applied Biosystems)
- dATP 100 mM (Invitrogen)
- dCTP 100 mM (Invitrogen)
- dGTP 100 mM (Invitrogen)
- dTTP 100 mM (Invitrogen)

-Reverse Transcriptase enzyme (MuLV RTR, *murine leukemia virus reverse transcriptase*) (Applied Biosystems)

- RNA samples isolated from PBMCs

Isolated total RNA was reverse transcribed to cDNA using iScript® cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's protocol. The iScript reverse transcriptase, RNase H+ enzyme, is a modified MMLV-derived reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA (100 fg to 1 µg of total RNA). The enzyme is provided preblended with RNase inhibitor. The iScript reaction mix consists of the unique blend of oligo(dT) and random hexamer primers.

To the total of 0.05 µg of total RNA (in a final volume of 15 µl) 5x iScript reaction mix (4 µl/sample) and iScript reverse transcriptase (1 µl/sample) was added. Complete reaction mix was incubated for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C.

Reagents:

- RT kit (iScript® cDNA synthesis kit) (Bio-Rad Laboratories)

Real-time PCR (qPCR)

QPCR was performed with use of SYBR® Green Detection Method. The principle behind the method is that as the PCR amplification process progresses, there is an increase in fluorescence from SYBR®Green dye. As the SYBR® Green dye binds to double-stranded amplicons, it undergoes a conformational change and emits fluorescence at a greater intensity.

For cDNA samples from different depots of adipose tissue, hypothalamus, liver and PBMCs, real-time PCR was performed by loading into PCR plate 2 µl of the dilution of RT product (1/5-1/10) and 9 µl of PCR mix. Per each sample, the mix consisted of: 5 µl of Power SYBR Green PCR Master Mix, 3.1 µl of RNA-ase free water and 0.475 µl of the each of the primers (forward and reverse). The concentration of the primers was dependent on mRNA abundance in a determined tissue. Real-time PCR was run using the Applied Biosystems StepOnePlus Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 min at 95°C followed by a total of 40 two temperature cycles (15 s at 95°C and 1 min at 60°C). For cDNA samples from stomach, 2 µl of the dilution of RT product (1/5) and 4.25 µl of PCR mix was loaded into PCR plate. Per each sample, the mix consisted of: 3.125 µl of Power SYBR Green PCR Master Mix, 0.5 µl of RNA-ase free water and 0.3125 µl of the each of the primers (forward and reverse). In this case, thermal cycling conditions were as follows: 7 min at 95°C followed by a total of 50 three-temperature cycles (15 s at 95°C, 20 s at 60°C and 40

s at 72°C). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.2.2), and the relative expression of each mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). Different housekeeping genes were used as internal controls for gene expression normalization depending on the tissue and the age and sex of the animals, according to their better suitability.

Reagents:

- RNA-ase free water (Sigma)
- Power SYBR Green PCR Master Mix (Applied Biosystems)
- Primers (provided by Sigma)

5. Microarray analysis

Two microarray analyses were performed. In the first microarray (microarray I), PBMCs samples from the offspring of control and calorie-restricted dams during lactation were used. This first microarray was conducted in the laboratory group of Prof. Dr. Jaap Keijer, in the Department of Human and Animal Physiology at Wageningen University, in the Netherlands. In the second (microarray II), PBMCs samples from the offspring of rat with free access to standard chow diet (control), the offspring of 20% calorie-restricted dams during the first 12 days of pregnancy (CR), and CR rats daily supplemented with physiological doses of leptin throughout lactation (CR-Leptin) were used. The second array was conducted in the laboratory of Molecular Biology, Nutrition and Biotechnology (LBNB) at the University of the Balearic Islands (UIB). In both cases, whole-genome microarray analysis was performed in RNA from PBMC samples. Agilent's platform for Two-Color Microarray-based Gene Expression Analysis was used, which employs cyanine 3- and cyanine 5-labeled targets to measure gene expression in experimental and control samples. According to manufacturer´s standard workflow, prior to array processing, RNA yield was quantified and its quality was determined. Then, the samples were adequately prepared (labeled, amplified and purified) for hybridization. Subsequently microarray was washed, scanned, and finally, microarray data were analyzed. Results obtained from microarray experiments were validated by RT-qPCR.

<u>Assessment of RNA quality and quantity</u>

In order to assess RNA quantity and to assure the high quality of RNA samples, RNA yield was quantified on the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and

its integrity was analyzed on Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip Kit (Agilent Technologies), according to manufacturer´s instruction.

Quantification of RNA samples with use of NanoDrop was performed as previously described (see section 3). Regarding integrity of RNA samples, the Agilent Bioanalyzer RNA assay enables the quality analysis of RNA using 1ul of sample. The NanoChip kit accommodates 12 sample wells, gel wells and a well for an external standard (ladder). These wells are interconnected through micro-channels. During chip preparation, the micro-channels are filled with a sieving polymer and fluorescence dye. Then, 1 ul of sample and ladder with marker are loaded in each well of kit. The assay is run on the Agilent 2100 Bioanalyzer instrument and utilizes the Agilent 2100 Expert Software to analyze and display results. An RNA Integrity Number (RIN score) is generated for each sample on a scale of 1-10 (1=lowest; 10=highest) as an indication of RNA quality. Only RNA samples with high RIN number (≥8) were used for microarray experiment.

Reagents:

- Quality assessment kit (RNA 6000 Nano LabChip Kit) (Agilent Technologies)

Sample preparation

In order to prepare labeling reaction, total RNA (40 ng in the microarray I or 80ng in the microarray II) in a total volume of 3.5 μl) from each sample was firstly reverse transcribed to complementary DNA (cDNA) using the Low Input Quick Amp Labeling Kit (Agilent Technologies), according to the manufacturer's protocol. Then, the volume of each cDNA sample was divided into two equal parts (10 μl each). Half of the cDNA sample was used for the linear amplification of RNA and labeling with cyanine-3 (Cy3) and the other with Cy5. For these reactions, half of the amounts indicated by the manufacturer were used (van Schothorst et al. 2007). Therefore, the mix used for transcription consisted of 0.375 μl nuclease-free water; 1.6 μl 5x transcription buffer; 0.3 μl 0.1M dithiothreitol (DTT); 0.5 μl nucleoside triphosphate (NTP) mix; 0.105 μl T7 RNA Polymerase Blend; 0.2 μl cyanine 3-CTP or cyanine 5-CTP, in a total volume of 3 μl. Prepared transcription mix (3 μl) was then added to 10 μl cDNA, and the transcription and labeling were carried out at 40°C for 2h. Subsequently, the labeled cRNA samples were purified using Rneasy Mini Spin Columns Kit (Qiagen). Specific activity (dye incorporation) and cRNA concentration was measured using the ''microarray measurement mode'' of the Nanodrop spectrophotometer (NanoDrop Technologies). Only samples with the highest yield (≥0.600 in the microarray I and >0.825

µg in the microarray II) and the highest specific activity (≥6.0 and >8.0 pmol Cy3 or Cy5 per µg of cRNA, in the microarrays I and II, respectively) were used for hybridization.

Reagents:

- Reverse transcription and labeling kit (Low Input Quick Amp Labeling Kit) (Agilent Technologies)
- Purification kit (Rneasy Mini Spin Columns Kit) (Qiagen)

Hybridization

Hybridization was performed using the Gene Expression Hybridization Kit (Agilent Technologies). All Cy3 cRNA's were pooled to serve as standard reference pool, while samples labeled with Cy5 served as a target samples. Per each sample, the mix prepared for hybridization consisted of linearly amplified cRNA (600-800 ng) labeled with Cy5, linearly amplified cRNA (600-800 ng) labeled with Cy3, 11 µl 10x Blocking Agent and 2.2 µl 25x Fragmentation Buffer, in a total volume of 55 µl. Then, the samples were incubated at 60°C for 30 min, and the fragmentation was stopped by adding 55 µl 2x GEx Hybridization Buffer HI-RPM. 100 µl of hybridization solution was dispensed into the gasket slide and assembled to the gene expression microarray slide (gasket slide/4 microarrays). The slides were hybridized on 4x44K G4131F Whole Rat Genome Agilent Microarrays (Agilent Technologies) for 17h at 65°C in hybridization chambers in an oven rotating at 10 rpm (Agilent Technologies)

Reagents:

- Hybridization Kit (Gene Expression Hybridization Kit) (Agilent Technologies)

Microarray wash

After hybridization, the arrays were washed using Gene Expression Wash Buffer Kit (Agilent technologies). The arrays were rinsed in GE Wash Buffer 1 for 1 min, GE Wash Buffer 2 for 1 min, followed by acetonitrile for 10 s and finally with a Stabilization and Drying Solution for 30 s at room temperature, according to the manufacturer's protocol (Agilent Technologies).

Reagents:

- Gene Expression Wash Buffer Kit (Agilent technologies)

- Stabilization and Drying Solution (Agilent Technologies)
- Acetonitrile (Sigma)

Scanning and microarray data analysis

The arrays were scanned with an Agilent Microarray Scanner (Agilent Technologies). Scanned images were examined for visible defects and proper grid alignment. The intensities of the signals from each spot were quantified, and the raw data were extracted using Feature Extraction Software (Agilent Technologies). Background correction and normalization were performed with use of GeneMaths XT 2.12 (Applied Mathematics) or within a suite of web tools for microarray data analysis - Babelomics (http://www.babelomics.org).

To search for differential gene expression between groups of animals two-way analysis of variance (ANOVA) was performed with SPSS v19.0 for Windows or the limma package from Bioconductor (limma t-test) implemented into Babelomics web platform. At any rate, *p*-values and fold changes (FC) were also provided. The threshold of significance for these statistical tests was set at $p \leq 0.01$. Subsequently, a statistically generated list of genes was manually analyzed in regard to their biological information, obtained with the use of available databases (Genecards, KEGG, NCBI, Reactome, UniProt, USCN, WikiPathways) based on key biological domains, such as molecular function and biological process. Some of these processes overlapped, thus they were collected, renamed and all the genes were assigned into several biological processes according to their function.

Validation of microarray data

To validate microarray data, mRNA expression levels of selected genes were measured by RT-qPCR in the same PBMC RNA samples. For both microarray experiments this step was performed in the LBNB at UIB, according to the protocols described in the section 4 (RT-qPCR analysis).

6. Protein extraction and quantification

The concentration of proteins in the samples was measured in tissue homogenates using Bradford method (Bradford 1976), with the application of 0.1% bovine serum albumin (BSA) as a standard (standard curve: 0-10 µg).

The Bradford method is based on the binding of the dye Coomassie Blue G250 to protein. Upon binding of the dye to protein the blue form of dye is generated with an increase in absorption at 595 nm. Thus, the quantity of protein can be estimated by determining the amount of dye in the blue ionic form. This is usually achieved by measuring the absorbance of the solution at 595 nm.

Accurately weighed tissue samples were homogenized at 4°C in the adequate volume of PBS buffer (supplemented with protease inhibitors). The volume of buffer added depended on the type of tissue (1:3 (w:v) for white adipose tissue, 1:10 (w:v) for brown adipose tissue and 1:20 (w:v) for liver). Completely homogenized tissues were centrifuged at 7500*g* for 2 min at 4°C, and then the supernatant containing proteins was collected to determine their concentration. Into the wells of standard ELISA plate 5µl of sample, blanc, or a different volumes of standard BSA were loaded, and then 250 µl of Bradford Reagent was added. After 2 min incubation absorbance was red at 595 nm versus water as the reference, using spectrophotometer Tecan Sunrise Absorbance Reader.

Reagents:

- Protease inhibitors: 10µl/ml phenylmethylsulfonyl fluoride (PMSF) (Sigma), 1µl/ml leupeptin (Sigma) and 1µl/ml aprotinin (Sigma)
-PBS buffer (pH 7.4): 137mM NaCl (Pancreac), 2.7mM KCl (Pancreac), 10mM Na_2HPO_4 (Pancreac), adjusted pH 7.4 and filled up to 1 liter with distilled water
- Bradford reagent: 100 mg of Coomassie Blue G250 (Sigma) dissolved in 50 ml of 95% ethanol, mixed with 100 ml of 85% phosphoric acid (Pancreac) and filled up to 1 liter with distilled water
- Bovine Serum Albumin (BSA) (Sigma) at 1 mg/ml is dissolved in PBS buffer and used as a standard

7. Western Blot

Western blotting is a method used to detect a target protein from a mixture of proteins. It uses gel electrophoresis to separate denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane, where they are detected using antibodies specific to the target protein. The membrane is then scanned and the bands are quantified.

Electrophoresis

Separation of proteins in an electric field by electrophoresis was performed with use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method employing Laemmli system (Laemmli 1970). The Laemmli SDS-discontinuous system refers to a discontinuous buffer system with SDS added to all buffers, to denature the proteins. SDS is an anionic detergent, meaning that dissolved molecules have a negative charge within a wide pH range. A polypeptide chain binds amounts of SDS in proportion to its relative molecular mass. The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode (positively-charged electrode) in an electric field. The Laemmli SDS-PAGE system is made up of four components, namely, the electrode buffer (the running buffer; Tris-glycine pH 8.3), the sample (prepared in Tris-Cl pH 6.8 buffer), the stacking gel (prepared in Tris-Cl pH 6.8 buffer) and resolving or separating gel (prepared in Tris-Cl pH 8.8 buffer). Preparation requires casting two different layers of acrylamide between glass plates. The lower layer (separating, or resolving, gel) is responsible for separating polypeptides by size, and the upper layer (stacking gel) includes the sample wells.

- Sample preparation

Prepared protein samples in tubes (the total concentration of proteins should be equal in each sample) were mixed with the required volume of sample buffer (1 μl of sample buffer for each 10 μg of protein) so that the ratio of protein/SDS remains 1:4. In order to denature proteins, the samples were heated in a boiling water bath for 2-4 min. Before the heating, the holes were punched in the top of tubes with needle to keep them from exploding. Then, samples were spun briefly to bring condensation to bottom.

Regents:

- Sample loading buffer, pH 6.8 (0.0625M Tris; 5% SDS; 10% -V/V- glycerol; 5% -V/V- 2-mercaptoethanol; 0.0025% bromophenol blue): 6.25ml 0.5M Tris-Cl, pH 6.8 (6 g Trisma base (Sigma) dissolved in 100 ml distilled water, pH adjusted to 6.8); 2.5 g SDS (Pancreac), 5 ml glycerol (Sigma), 2.5 ml 2-mercaptoethanol (Sigma), 0.125 ml 1% bromophenol blue (0.1 g of bromophenol blue (Sigma) dissolved in 10 ml bidistilled water), filled up to 500 ml with distilled water.

- Gel preparation

Two clean and dry glass plates were put together to and aligned in clamp. Then, resolving and stacking gels were prepared. An appropriate strength of gels was chosen based on the

size of proteins selected for separation (for larger size of proteins lower percentage of resolving gel was used).

- Resolving gel (10% acrylamide; 0.1%, SDS; 0.375M Tris (pH 8.8); 0.05% TEMED; 0.05% ammonium persulfate). For gel thickness 1.5 mm: 15 ml 30% acrylamide solution; 22.5 ml buffer Tris-SDS (pH 8.8); 7.5 ml bisdistilled water; 250 µl 10% ammonium persulfate (APS); 25 µl TEMED. TEMED (in hood) and APS were added at the end, and the solution was mixed well without forming bubbles.
- Stacking gel (4% acrylamide; 0.1%, SDS; 0.125M Tris (pH 6.8); 0.1% TEMED; 0.05% ammonium persulfate). For gel tickness 1.5 mm: 1.5 ml acrylamide solution (30%); 7.5ml buffer Tris-SDS (pH 6.8); 6 ml bisdistilled water; 200 µl 10% ammonium persulfate (APS); 20 µl TEMED. TEMED (in hood) and APS were added at the end, and the solution was mixed well without forming bubbles.

Prepared resolving gel was poured with pipette immediately onto mold, the overlay of distilled water was added on the top, and the gel was left to polymerize (at least 30 min) at room temperature. After polymerization, the water overlay was removed carefully to leave the surface dry enough. Then, stacking gel was poured with pipette on top of mold and the comb was inserted between the two glass plates avoiding air bubbles, and the gel was left to polymerize (at least 15 min) at room temperature. After polymerization, the comb was pulled out gently and the wells were washed with Tris- glycine (running buffer).

Reagents:

- Gel buffers:
- Buffer Tris-SDS, pH 6.8 (0.25M Tris; 0.2% SDS): 15.14 g Trisma Base (Sigma), 1 g SDS (Pancreac), pH adjusted to 6.8 and filled up to 500 ml with distilled water.
- Buffer Tris-SDS, pH 8.8 (0.75M Tris; 0.2% SDS): 45.41 g Trisma Base (Sigma), 1 g SDS (Pancreac), pH adjusted to 8.8 and filled up to 500 ml with distilled water.
- 30% Acrylamide solution (Bio-Rad)
- 10% Ammonium persulfate (APS): 1 g APS (Sigma) dissolved in 10 ml distilled water with gentle agitation
-TEMED (N,N,N′,N′-Tetramethylethylenediamine) (Sigma)

- Loading samples and running the gel

The gel was transferred to tank apparatus and the tank was filled inside and outside with Tris-glycine (running buffer). Then, the entire sample and molecular weight standard were loaded

into appropriate wells. The lid on the electrophoresis unit was placed (the red (positive) electrode and the black (negative) electrode aligned to the appropriately colour cord).The gel was run at 100V through the stacking part of the gel and then the volts were turned down to 75V, after the proteins have gone through the stack and are migrating through the resolving gel. The migration was allowed to continue until the blue dye front was at the end of the glass plates, but did not migrate off the gel.

Reagents:

- Running electrophoresis buffer: Buffer Tris-Glycine, pH 8.3 (0.025M Tris; 0.192M Glycine; 0.1% SDS;): 15.14 g Trisma base (Sigma), 72 g glycine (Sigma), 5 g SDS (Pancreac), pH adjusted to 8.3 and filled up to 5 liters with distilled water.
- Molecular weight standard (Precision Plus Protein™ Dual Color Standards) (Bio-Rad)

Electroblotting

The proteins were transferred to a nitrocellulose membrane (0.45 µm por size to transfer separated proteins with size 30-250 kDa) (Bio-Rad) with use of semy-dry electroblotter, Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). The different sheets of Whatman paper nº3 were placed on a graphite plateavoiding the formation of bubbles and wrinkles as follows: 6 sheets wet in 0.3M Tris buffer, 3 sheets wet in 0.025M Tris buffer, nitrocellulose membrane wet with 0.025M Tris buffer, polyacrylamide gel obtained after electrophoresis performed, 9 sheets wet with the cathode buffer, and finally, the second graphite plate was placed. Electroblotter run at 0.20-0.25 A for 2-3h.

Reagents:

- Cathode buffer, pH 9.4: 0.445 g β-alanine (Sigma), 2.301 g Trisma base (Sigma), 100 ml methanol absolute (Pancreac), filled up to 500 ml with distilled water
- Buffer 0.025M Tris, pH 10.4: 1.51 g Trisma base (Sigma), 100 ml methanol absolute (Pancreac), filled up to 500 ml with distilled water
- Buffer 0.3M Tris, pH 10.4: 18.17 g Trisma base (Sigma), 100 ml methanol absolute (Pancreac), filled up to 500 ml with distilled water

Labelling and detection:

The membrane was blocked in Odyssey® Blocking Buffer overnight at 4°C. Then, the membrane was incubated with primary antibody (diluted 1:2000-1:4000 in TBS with 0.1%

BSA, 0.1% sodium azide and 0.1 - 0.2% Tween-20, to lower non-specific binding) for 60 min at room temperature with gentle shaking. The membrane was washed 3 times in TBS1x (when phospho-specific antibodies were used) or PBS 1x-Tween buffer, for 15, 5 and 5 min at room temperature, with gentle shaking, and then the buffer was discarded. Subsequently, the membrane was incubated with secondary antibody the fluorescently labeled secondary antibody (diluted 1:20000 in Odyssey® Blocking Buffer and TBS 1x or PBX 1x mixed 1:1, with 0.1% BSA, 0.1% sodium azide and 0.1 - 0.2% Tween-20) for 30-60 min at room temperature with gentle shaking, and protected from light. Secondary antibody was prepared in the same host species that the first antibody. The membrane was washed 3 times in TBS 1x or PBS 1x-Tween buffer, for 15, 5 and 5 min at room temperature, with gentle shaking, and then the buffer was discarded. Finally, the membrane was rinsed with TBS 1x or PBS 1x to remove residual Tween-20.

Reagents:

- Odyssey® Blocking Buffer (LI-COR Biosciences)
- TBS 10x wash buffer, pH 7.4: 500 ml 1M Tris-Cl, pH 7.4, 300 ml 5M NaCl, filled up to 1 liter with distilled water, autoclaved
-TBS 1x-Tween 20 wash buffer: TBS 10x buffer diluted 1:10 with distilled water, 1 g Tween-20 (Sigma)
- PBS 10x wash buffer, pH 7.4: 80 g NaCl (Pancreac), 2 g KCl (Pancreac), 11.5 g Na_2HPO_4 (Pancreac), 2 g KH_2PO_4 (Pancreac), pH adjusted to 7.4 and filled up to 1 liter with distilled water, autoclaved
-PBS 1x-Tween 20 wash buffer: PBS 10x buffer diluted 1:10 with distilled water, 1 g Tween-20 (Sigma)
- Bovine Serum Albumin (BSA) (Sigma)
- Sodium azide (Sigma)
- Secondary antibody: IRDye 800CW Goat anti-Rabbit IgG (H+L), IRDye 680LT Goat anti-Mouse IgG (H+L) (LI-COR Biosciences)

Scanning and quantification

The membrane was scanned on Odyssey Scanner with Infrared Imaging System (LI-COR Biosciences) in the appropriate channels IR-dyed (700 nm green channel when anti-mouse secondary antibody was used or 800 nm red channel when anti-rabbit secondary antibody was used). The quantification of all sample bands was performed using the Odyssey software

V3.0 (LI-COR Biosciences). Individual features (squares) that surrounded all the fluorescent dots or bands in the image were drawn. Then, the image data (including raw intensity, average intensity and integrated intensity) within the feature were quantified automatically. For more reliable results in the quantification the option for Integrated Intensity (the sum of the intensity values for all pixels enclosed by a feature, multiplied by the area of the feature (mm^2)) was chosen. Since background pixels should not be part of this calculation, background was calculated (Lane Background method) and subtracted.

Loading control

For the evidence of correct loading and blotting of proteins, incubation with β-actin antibody or staining with amido-black was performed.

In order to stain with β-actin antibody, the membrane was incubated with mouse monoclonal anti-β-actin antibody, diluted 1:400 (Cell Signaling). Staining with β-actin was performed according to the protocol described above in the section for labelling and detection of specified proteins. The membrane was scanned, β-actin bands were quantified (see the section Scanning and quantification), and then, the band intensity for the specific protein was normalized to that of β-actin.

In order to stain with amido-black, Amido Black Staining Solution (Sigma) was prepared by diluting Amido Black 1X Staining Solution with distilled water 1:1, according to manufacturer's instruction. After transferring proteins onto nitrocellulose membrane, the membrane was immersed in sufficient Amido Black 1X Staining Solution to cover, stained for about 1 min, and rinsed with deionized water until the lighter color of background was obtained. Stains were visualized using ChemiGenius connected with a program GeneSnap.

Reagents:

- Amido black staining solution (Sigma)

8. Morphometric analysis

For morphometric analysis,fragments of inguinal white adipose tissue and the entire brain samples were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer (pH=7.4) at 4°C for 24h, then washed and stored in 0.1 M phosphate buffer (pH=7.4) until posterior analysis. Subsequently, samples were dehydrated in graded series of ethanol (75, 90 and 100%), cleared in xylene and embedded in paraffin. Then, tissues in the fixed blocks

were cut (5 μm thick) using a microtomeand mounted on Super-Frost/Plus slides. In the fixed brains a coronal block containing the hypothalamus was cut. The sections were stained with hematoxylin-eosin solution for 5 min. Stained sections were analyzed with use of optical microscope equipped with camera digital. Microscope images of the sections were captured at 10x magnification and analysis were performed using AxioVision40V 4.6.3.0. Software (Carl Zeiss, Imaging Solutios). From the adipose tissue sections images of adipocites were obtained and their diameter was determined. From the sections containing hypothalamus, images of arcuate and paraventicular nucleus were obtained, in which the area occupied by each of nuclei was demarcated and the number of hematoxylin/eosin-stained cells was also measured.

Reagents:

- 4% Paraformaldehyde (Sigma)
- 0.1 M phosphate buffer (pH=7.4): 0.2M phosphate buffer (3.25 g $NaH_2PO_4 \cdot 2H_2O$ (Pancreac); 11.24 g Na_2HPO_4 (Pancreac) dissolved in 1 liter of distilled water, pH adjusted to 7.4) diluted in distilled water 1:1.
- Paraffin wax (Sigma)
- Ethanol absolute (Pancreac)
- Xylene (Pancreac)
- Hematoxylin (Pancreac)
- Eosin (Pancreac)

9. Immunohistochemical analysis

Immunohistochemical demonstration of NPY in ARC and PVN, as well as TH in rWAT was performed with the avidin-biotin peroxidise (ABC) method (Paxinos and Watson 1998; Hsu et al. 1981).

<u>NPY in ARC and PVN</u>

Sections were incubated sequentially at room temperature in the following solutions: 0.3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase; Citrate-based solution (pH=6) in microwave oven for 15 min and 20 min on ice for antigen retrieval; 2% goat normal serum in phosphate buffered saline (PBS) (pH 7.4-7.6) 0.1% Triton X-100 for 20 min to reduce non-specific background staining prior to incubation with primary antibody (polyclonal anti-NPY antibody produced in rabbit (N9528, Sigma-Aldrich), 1:800 in PBS

0.1% Triton X-100 with 1% BSA for 1 h and 15 min at 37°C); biotinylated goat antirabbit IgG (Vector Laboratories) 1:200 in PBS 0.1% Triton X-100 with 1% BSA for 1 h at room temperature; peroxidase-labeled ABC reagent (Vectastain ABC kit) in PBS for 30 min at room temperature and Fast 3,3-diaminobezidine tablet, DAB in PBS 0.1% Triton X-100 for 3 min in dark room for enzymatic development of peroxidase. Subsequently, slides were washed with deionized water, dehydrated with increasing concentrations of ethanol and xylene, mounted with Eukitt and cover-slipped. Negative controls were performed by omission of primary antibody. Capture of images (at a 10x magnification) and analysis were performed using AxioVision40V 4.6.3.0. Software (Carl Zeiss, Imaging Solutios GmbH).

Measurement of the number of immunoreactive NPY (NPY^+) neurons in ARC and the area occupied by NPY^+ fibers in PVN, were performed in 3 digitalized images/animal from the ARC (−2.3 to −3.3 mm posterior to Bregma) and from the PVN (−1.6 to −1.88 mm posterior to Bregma) according to published coordinates (Paxinos and Watson 1998) and with the help of hematoxylin/eosin staining. Image analysis from all groups was examined by two independent researchers in a blind fashion.

TH in iWAT

Sections were incubated sequentially at room temperature in the following solutions: 0.3% hydrogen peroxide in distilled water for 10 min to block endogenous peroxidase; 2% goat normal serum in phosphate buffered saline (PBS) (pH 7.4-7.6) for 20 min to reduce non-specific background staining prior to incubation with primary antibody (anti-TH rabbit polyclonal antibody (AB1542, Chemicon International) dilution 1:250 in PBS for 24h at 4°C; biotinylated goat antirabbit IgG (Vector Laboratories) 1:200 in PBS for 30 min at room temperature; peroxidase-labeled ABC reagent (Vectastain ABC kit) 1:100 in PBS for 30 min at room temperature and 3,3-diaminobezidine tablet, DAB in distilled water for 3 min in dark room for enzymatic development of peroxidase. Subsequently, slides were washed with distilled water, dehydrated with increasing concentrations of ethanol and xylene, mounted with Eukitt and cover-slipped. As a positive controls served TH stained sections of brown adipose tissue (BAT). Capture of images (at a 10x magnification) and analysis were performed using AxioVision40V 4.6.3.0. Software (Carl Zeiss, Imaging Solutios GmbH).

Measurement of the immunoreactive TH (TH^+) area in rWAT was performed in 2 digitalized images/animal and with the help of hematoxylin/eosin staining. Image analysis from all groups was examinedin a blind fashion.

Reagents:

- Eukitt (Pancreac)
- 0.3% hydrogen peroxide in methanol (Sigma)
- Phosphate buffered saline (PBS) (pH 7.4-7.6): 137mM NaCl (Pancreac), 2.7mM KCl (Pancreac), 10mM Na_2HPO_4 (Pancreac), adjusted pH 7.4 and filled up to 1L with distilled water
- 3,3-diaminobezidine (DAB) (Sigma)
- ABC reagent (Vectastain ABC kit) (Vector)
- Triton X-100 (Sigma)
- Bovine Serum Albumin (BSA) (Sigma)
- Biotinylated goat antirabbitIgG (Vector)
- Ethanol absolute (Pancreac)
- Xylene (Pancreac)
- 0.01M citrate (ph=6): 1.92 g of citric acid (C6H8O7) dissolved in 900 ml of distilled water adjusted pH 6.0 and filled up to 1L with distilled water

10. Determination of adiponectin levels

Determination of adiponectin content in plasma was performed with use of quantitative sandwich enzyme immunoassay technique applied in Rat Total Adiponectin/Acrp30 Quantikine ELISA Kit (R&D systems).

The assay was performed according to the manufacturer´s instructions. Firstly, 50 µl of Assay Diluent was loaded into each well of microplate supplied. This microplate is pre-coated with a monoclonal antibody specific for rat adiponectin. Then, 50 µl of sample or different dilutions of Standards (standard curve) were pipetted into the wells, and plate was incubated at room temperature for 1h on a horizontal orbital microplate shaker. During incubation process, any rat adiponectin present was bound by the immobilized antibody. Subsequently, any unbound substances were aspirated and washed with Wash Buffer, and 100 µl of Conjugate (an enzyme-linked polyclonal antibody specific for rat adiponectin) was added to each well, and plate was incubated at room temperature for 1h on a horizontal orbital microplate shaker. Following a wash to remove any unbound antibody-enzyme reagent, 100 µl of Substrate Solution was added to the wells, and plate protected from light was incubated at room temperature for 30 min on the benchtop. During the incubation process the enzyme reaction yielded a blue product that turned yellow when 100 µl of the Stop Solution was

added. The intensity of the colour measured was in proportion to the amount of rat Adiponectin bounded in the initial step. The absorbance was then read at 450 nm with wavelength correction at 540 nm using spectrophotometer Tecan Sunrise Absorbance Reader.

Reagents:

- Adiponectin kit (Rat Total Adiponectin/Acrp30 Quantikine ELISA Kit) (R&D systems)

11. Determination of insulin levels

Determination of insulin in plasma was performed with use of quantitative two-site sandwich enzyme immunoassay technique applied in Rat Insulin Enzyme-linked Immunosorbent Assay ELISA Kit (Mercodia). Insulin in plasma of young rats (until the age of 1 month) was quantified with use of Ultrasensitive Rat Insulin ELISA Kit (Mercodia).

The assay is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to micro-titer well. A simple washing step removes unbound enzyme labeled antibody. The bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

The assay was performed according to the manufacturer´s instructions. After preparation of adequate volume of Enzyme Conjugate 1X solution (dilution of Enzyme Conjugate11X in Enzyme Conjugate buffer 1+10), depending on the number of samples used, 10 µl (25 µl in case of ultrasensitive insulin kit) of the sample or each of the Calibrators were loaded into each well of microtiter. Then, 100 µl of Enzyme Conjugate 1X solution was added and plate was incubated on a plate shaker (700-900 rpm) for 2h at room temperature. During incubation insulin in the sample reacted with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to microtiter well. Subsequently, unbound enzyme labeled antibody were removed by aspiration and washing with Wash Buffer, and 200 µl of Substrate TMB was added into each well, followed by 15 min incubation at room temperature. Then, 50 µl of Stop Solution was added to each well, plate was placed on the shaker for approximately 5 s to ensure mixing and optical density was read at 450 nm using spectrophotometer Tecan Sunrise Absorbance Reader.

Reagents:

- Insulin kit (Insulin Enzyme-linked Immunosorbent Assay ELISA Kit or Ultrasensitive Rat Insulin ELISA Kit) (Mercodia)

12. Determination of leptin concentration

Determination of the concentration of leptin in plasma and in stomach homogenates was performed with use of quantitative sandwich enzyme immunoassay technique applied in kit Quantikine™ Mouse Leptin Inmunoassay (R&D Systems). In addition, this kit was also used as a control of adequate concentrations of leptin used to orally treat animals.

This assay is prepared to measure mouse leptin, however, it also demonstrates significant cross-reactivity with rat leptin, and has been validated to determine relative mass values for natural rat leptin, measured as mouse leptin equivalent.

The assay was performed according to the manufacturer´s instructions. Firstly, 50 µl of Assay Diluent RD1W was loaded into each well of microplate supplied. This microplate is pre-coated with an affinity purified polyclonal antibody specific for mouse leptin. Then, 50 µl of sample or different dilutions of Standards (standard curve) were pipetted into the wells, and plate was incubated at room temperature for 2h at room temperature. During incubation process, any mouse leptin present is bound by the immobilized antibody. Subsequently, any unbound substances were aspirated and washed with Wash Buffer, and 100 µl of Mouse Leptin Conjugate (an enzyme-linked polyclonal antibody specific for mouse leptin) was added to each well, and plate was incubated for 2h at room temperature. Following the wash to remove any unbound antibody-enzyme reagent, 100 µl of Substrate Solution was added to each well, and plate was incubated at room temperature for 30 min. During the incubation process the enzyme reaction yielded a blue product that turned yellow when 100 µl of the Stop Solution was added. The intensity of the colour measured was in proportion to the amount of mouse leptin bounded in the initial step. The absorbance was then read at 450 nm with wavelength correction at 540 nm using spectrophotometer Tecan Sunrise Absorbance Reader.

Reagents:

- Leptin kit (Quantikine™ Mouse Leptin Inmunoassay (R&D Systems)

13. Determination of non-esterified fatty acid levels

Determination of non-esterified fatty acids (NEFA) content in plasma was performed with use of enzymatic colorimetric method applied in kit NEFA C (Wako).

This enzymatic method relies upon the acylation of coenzyme A (CoA) by the fatty acids in the presence of added acyl-CoA synthetase (ACS). The acyl-CoA thus produced is oxidized by added acyl-CoA oxidase (ACOD) with generation of hydrogen peroxide, in the presence of peroxidase (POD) permits the oxidative condensation of 3-methy-N-ethyl-N(β-hydroxyethyl)-aniline (MEFA) with 4-aminoantipyrine to form a purple colored adduct which can be measured colorimetrically at 550 nm. The intensity of this pigment is proportional to the concentration of free fatty acids in the sample. Ascorbic acid is removed by ascorbate oxidase from the sample.

Firstly, the contents of the reagents of the kit were dissolved according to the manufacturer´s instructions, to prepare colour test solutions. Into each well of standard ELISA plate (96 wells) 4.92 µl of sample or different volumes of NEFA C Standard (standard curve) were loaded. Then, 98.4 µl of color reagent solution A was added into each well and plate was incubated for 10 min at 37°C. Subsequently, 196 µl of color reagent solution B was added into each well and, the plate was gently mixed and then incubated for 10 min at 37°C. Plate was brought to room temperature and the absorbance was read at 550 nm versus water as the reference, using spectrophotometer Tecan Sunrise Absorbance Reader.

Reagents:

- Non-esterified fatty acids (NEFA) kit (NEFA C) (Wako)

14. Determination of triglycerides levels

Determination of triglyceride content in plasma was performed with use of enzymatic colorimetric method applied in kit Serum Triglyceride Determination (Sigma).

The procedure involves enzymatic hydrolysis by lipase of the triglycerides to glycerol and free fatty acids. The glycerol produced is then measured by coupled enzyme reactions. Triglyceride assay enzymatic reactions are as follows: Triglycerides are first hydrolyzed by lipoprotein lipase to glycerol and free fatty acids. Glycerol is then phosphorylated by adenosine-5´-triphosphate (ATP) forming glycerol-1-phosphate (G-1-P) and adenosine-5´-

diphosphate (ADP) in the reaction catalyzed by glycerol kinase (GK). G-1-P is then oxidized by glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H2O2). Peroxidase (POD) catalyzes the coupling of H_2O_2 with 4aminoantipyrine (4-AAP) and sodium *N*-ethyl-*N*-(3-sulfopropyl) *m*-anisidine (ESPA) to produce a quinoneimine dye that shows an absorbance maximum at 540 nm. The increase in absorbance at 540 nm is directly proportional to triglyceride concentration of the sample. Free, endogenous glycerol may be measured using the same coupled enzyme reactions without the initial lipase hydrolysis. The reaction sequence would be the same except for the lipolytic reaction. The increase in absorbance at 540 nm is then directly proportional to glycerol concentration of the sample.

The assay was performed according to the manufacturer´s instructions, with some modifications in order to use the kit in a microplate format. Into each well of standard ELISA plate (96 wells) 240 µl of Free Glycerol Reagent was loaded. Then, 3 µl of sample or different dilutions of Glycerol Standard in water (standard curve) was loaded. Subsequently, plate was incubated for 5 min at 37°C and the initial absorbance was read at 540 nm (IA) versus water as the reference, using spectrophotometer Tecan Sunrise Absorbance Reader. After determination of glycerol concentrations, 60 µl of Triglyceride Reagent was loaded into each well and plate was incubated for 5 min at 37°C. The final absorbance was red at 540 nm (FA) versus water as the reference to determine total triglycerides concentration. To calculate the concentration of triglycerides in the sample the content of glycerol was subtracted from the concentration of total triglycerides.

Reagents:

- Triglycerides kit (Serum Triglyceride Determination kit) (Sigma)

15. Determination of triiodothyronine (T3) levels

Determination of triiodothyronine (T3) in plasma was performed with use of quantitative enzyme immunoassay technique applied in Total T3 ELISA Kit (DRG International, Inc.).

The assay is based on the principle of competitive binding. The microtiter wells are coated with a polyclonal goat-anti-mouse antibody. Standards, controls and specimen serum incubate together with Assay Reagent containing monoclonal anti-T3 antibodies. In the following incubation with Conjugate the endogenous T3 of a specimen sample competes with the T3-horseradish peroxidase conjugate for a limited number of insolubilized binding sites.

After incubation the unbound conjugate is washed off. The amount of bound peroxidase conjugate is inversely proportional to the concentration of T3 in the sample. After addition of the substrate solution, the intensity of color developed is inversely proportional to the concentration of T3 in the specimen sample.

The assay was performed according to the manufacturer´s instructions. Firstly, 50 µl of sample or different concentrations of Standard (standard curve) were dispensed into each well of the microtiter, then, 50 µl of Assay Reagent was added to each well, and plate was thoroughly mixed for 10 s and incubated for 30 min at room temperature. Subsequently, 50 µl of Enzyme Conjugate was dispensed into each well, thoroughly mixed for 10 s and incubated for 30 min at room temperature. Following the wash to remove the unbound conjugate, 100 µl of Substrate Solution was added to each well, and a plate was incubated for 10 min at room temperature. The enzymatic reaction was stopped by adding 100 µl of Stop Solution to each well, and optical density was read at 450 nm using spectrophotometer Tecan Sunrise Absorbance Reader.

Reagents:

- T3 kit (Total T3 ELISA Kit) (DRG International, Inc.)

16. Hepatic lipids extraction and quantification

The extraction of lipids from liver samples was performed with Folch *et al*. (Folch et al. 1957) extraction procedure.

The liver tissue weighed precisely (0.3 g) was homogenized with 5 ml of chloroform-methanol reagent (2:1 by volume). The exact weight of tissue was recorded for the following calculations. The tissue was disrupted using Potter-Elvehjem homogenizer with a motorized PTFE pestle, and the homogenate was filtrated (funnel with a folded filter paper) to recover the liquid phase. Then, the total volume of the mixture was filled up to 10 ml with chloroform-methanol reagent (2:1 by volume) and the mixture was washed with 2 ml of 0.45% sodium chloride (NaCl) solution. After dispersion, the whole mixture was agitated vigorously 2 min in an orbital shaker and centrifuged at low speed (3000 rpm) for 10 min at room temperature to separate the two phases. The upper phase (methanol and impurities) was removed, the lower phase (chloroform with lipids) was filled up to 10 ml with pure methanol, and washed with 2 ml of 0.9% NaCl solution. Subsequently, the whole mixture was agitated vigorously 2 min in an orbital shaker and centrifuged at low speed (3000 rpm) for 10 min at

room temperature. After centrifugation and siphoning of the upper phase, the lower phase (chloroform with lipids) was filled up to 10 ml with chloroform-methanol reagent (2:1 by volume). 5 ml of the extract was then leaved in a glass vial at 60^0C for 24h to evaporate chloroform. The dry and empty glass vials and vials with lipid extracts were weighed, and the total lipid content was calculated using the following formula:

Lipid content [mg/g liver] = ((extract weight [mg]/extract weight [ml]) *10)/sample weight [g],

where,

extract weight [mg] = (weight of the empty glass vial [g] - weight of the glass vial with the extract after evaporation [g]) * 1000;

extract weight [ml] = 5 ml;

sample weight [g] – weight of liver sample taken for homogenization (the recommended amount of sample is ~0.3 g, although the exactly amount taken was noted).

Knowing the weight of the liver of each animal, the content of lipid in the whole liver was calculated.

Reagents:

- Chloroform/methanol (2/1): 2 parts of chloroform (Sigma) diluted with 1 part of methanol absolute (Pancreac)
- Sodium chloride (NaCl) 0.45%: 2.25 g NaCl (Pancreac) dissolved in 500 ml of distilled water
- Sodium chloride (NaCl) 0.90%: 4.5 g NaCl (Pancreac) dissolved in 500 ml of distilled water

17. Statistical analysis

The statistical analysis of microarray data has been described in the section referred to microarray data analysis. The methods used for statistical analyses were chosen based on the experimental questions involved. To study individual differences between experimental groups, multiple comparisons were assessed by repeated measures, one- and two-way analysis of variance (ANOVA), followed by least significance difference (LSD) *post hoc* test in some cases. The relationship between random variables was estimated with use of Person´s correlation coefficient. Single comparisons between groups were assessed by Student's *t* test and Paired *t* test. Threshold of significance was set at $p<0.05$. All the analyses were performed with SPSS Statistics (SPSS, Chicago, IL).

18. References

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