Universitat de les
Illes Balears

DOCTORAL THESIS

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METABOLIC PROGRAMMING ASSOCIATED TO GESTATIONAL MILD CALORIE RESTRICTION IN RATS AND ITS POTENTIAL REVERSIBILITY BY LEPTIN

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Doctoral Programme of Nutrigenomics and Personalized Nutrition

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WE DECLARE:

That the thesis entitled *Metabolic programming associated to gestational mild calorie restriction in rats and its potential reversibility by leptin,* presented by Nara Jimena Szostaczuk to obtain a doctoral degree, has been completed under our supervision and meets the requirements to opt for an European Doctorate Mention.

For all intents and purposes, we hereby sign this document.

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Palma de Mallorca, 9th June 2017

To my family, specially to my grandfathers

Juan and Kazimierz

Because of your love, I become the person who I am

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Abbreviations

List of most common abbreviations used throughout the Thesis:

ABC: Avidin-biotin peroxidase
AC: Adenylate cylcase
ACC: Acetyl-CoA carboxylase
AgRP: Agouti-related peptide
AMPK: Monophosphate-activated protein kinase
ARC: Arcuate nucleus
ASP: Acylation stimulating protein
BDNF: Brain-derived neurotrophic factor
CART: Cocaine and amphetamine-regulated transcript
cDNA: Complementary DNA
CGI-58: Gene identification-58
CLD: Congenital leptin deficiency
CoA: Coenzyme A
CPT1: Carnitine palmitoyltransferase 1
CR: Caloric restriction
Cy3: Cyanine-3
Cy5: Cyanine-5 .
DG: Diacylglycerol
DIO2: Deiodinase iodothyronine type II
DMN: Dorsomedial nucleus
DNA: Desoxirribonucleic acid
DOHaD: Developmental origins of health and disease
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
ER: Endoplasmic reticulum
FAS: Fatty acid synthase
FATP: Fatty acid transport protein
FC: Fold change
G6P: Glucose 6-phosphate
G6Pase: Glucose-6-phosphatase
GHSR1a: Growth hormone secretagogue receptor 1a
GK: Glucokinase
GKRP: GK regulatory protein
GLUT4: Glucose transporter type 4
GOAT: Ghrelin O-acyltransferase
GSK-3: Glycogen synthase kinase 3
HF: High-fat
HOMA-IR: Homeostatic model assessment for insulin resistance
HSL: Hormone sensitive lipase
IL-6: Interleukin-6
InsR: Insulin receptor

JAKs: Janus kinases
LHA: Lateral hypothalamic area
LPL: Lipoprotein lipase
MCH: Melanin-concentrating hormone
MetS: Metabolic syndrome
MG: Monoacylglycerol
MGL: Monoacylglycerol lipase
mRNA: Messenger ribonucleic acid
NE: Norepinephrine
NEFA: Non-esterified fatty acids
NPY: Neuropeptide Y
ObRb: Long form leptin receptor
PAI-1: Plasminogen activator inhibitor-1
PBMCs: Peripheral blood mononuclear cells
PCA: Principal component analysis
PDH: Pyruvate dehydrogenase
PDK1: 3-phosphoinositide dependent protein kinase-1
PGC1A: Receptor-gamma coactivator 1 alpha
PI3-kinase: Phosphoinositide 3-kinase
PKA: Protein kinase A
POMC: Pro-opiomelanocortin
PPARα: Proliferator-activated receptor alpha
PPARγ: Peroxisome proliferator-activated receptor gamma
PVN: Paraventricular nucleus
q-PCR: Quantitative polymerase chain reaction
RTK: Tyrosine kinase receptor
SD: Standard diet
STAT3: Signal transducer and activator of transcript 3
T3: Triiodothyronine
T4: Thyroxine
TCA: Tricarboxylic acid
TG: Triacylglycerides
TNFα: Necrosis factor alpha
TyrOH: Tyrosine hydroxylase
UCP1: Uncoupling protein 1
VLDL: Very low-density lipoprotein
VMN: Ventromedial nucleus
WAT: White adipose tissue
WD: Western diet
WHO: World Health Organization

Abstract

Metabolic programming associated to gestational mild calorie restriction in rats and its potential reversibility by leptin

The prevalence of obesity is increasing globally and emerging in developing countries. The etiology of obesity is multifactorial, involving complex interactions among the genetic background and social and environmental factors, such as lifestyle and dietary habits. In addition, conditions during the first periods of life, particularly gestation and the immediate postnatal period, may program the development of tissues and organs and cause lifelong effects on metabolic and physiological functions, influencing the state of health. Considering the interest in obesity prevention, the study of metabolic programming effects in early stages of life and its potential reversion are important research objectives. Our research group has previously described that supplementation with physiological doses of oral leptin throughout the suckling period in rats have significant short- and long-term metabolic health benefits, such as a greater protection against diet-induced obesity and its metabolic-related disorders. In this line, more recent studies showed that leptin supplementation during lactation is able to revert the malprogrammed effects on hypothalamic and white adipose tissue structure and function observed at a juvenile age in the offspring of mild calorie-restricted dams during gestation. However, whether the beneficial effects of leptin are long-lasting, protecting against the acquired risk to obesity-related metabolic alterations in adulthood was still unknown.

In the present PhD thesis, ***we aimed to study in rats whether the metabolic alterations and obesogenic risk in adulthood associated to a mild (20%) calorie restriction during gestation may be prevented by leptin supplementation throughout the suckling period. Moreover, we also aimed to use this animal model to identify potential transcript-based biomarkers in peripheral blood mononuclear cells (PBMCs) in adulthood indicative of a higher risk to obesity-related metabolic alterations and sensitive to early nutritional intervention.***

The results shown in this doctoral thesis describe that leptin supplementation at physiological doses throughout lactation in rat pups prevents the dysmetabolic phenotype in adulthood associated to mild calorie restriction during pregnancy and improves metabolic adaptations to an obesogenic diet. The adult offspring of calorie restricted dams during gestation (CR) presented increased adiposity, along with higher feed efficiency, lower energy expenditure and a reduction in locomotive activity compared to control animals, suggesting an imbalance in energy homeostasis. Moreover, adult CR animals displayed higher HOMA-IR index and greater plasma leptin levels than controls. These alterations were mostly normalized in CR animals supplemented with oral leptin during the suckling period (CR-Leptin). CR animals also exhibited more severe signs of hepatic steatosis under western diet than CR-Leptin animals, indicative of an impaired adaptation to the hypercaloric diet and related to the reduced insulin sensitivity. Moreover, CR animals, but not CR-Leptin animals, showed indicators of lower white adipose tissue expansion capacity, together with a decreased

lipogenesis and lipolytic capacity, also in accordance with the diminished insulin sensitivity and leptin signaling. Leptin supplementation during lactation also reversed the decreased sympathetic drive to the stomach that suffered CR animals, particularly at a juvenile age, and also restored the diminished circulating ghrelin levels present in CR animals. This may account for the normalization of the leptin/ghrelin ratio, which is regarded as a clinical non-invasive marker of obesity-related metabolic alterations.

The above mentioned animal models (i.e. rats exposed to certain undernutrition during gestation and rats treated with leptin during lactation) have been used to identify a set of transcript-based biomarkers in PBMCs in adulthood as potential indicators of predisposition to alterations related to the metabolic syndrome and sensitive to its reversal by nutritional intervention in early life. Notably, among the genes identified, two of them are related to feeding control (*Agrp* and *Pomc*), and others with lipid metabolism, and with connections to insulin homeostasis, hepatic steatosis, and cardiovascular health (*Pcyt2*, *Rxrb*, *Insig1*, *Npc1*, *Slc27a4*, *Apoo*, *Pla2g2a* and *Sort1*).

All in all, results obtained in this thesis bring additional evidence supporting the relevance of the intake of appropriate amounts of leptin throughout lactation to reverse postnatal sequelae induced by deficient fetal nutrition, and hence could be worth being considered in the search of strategies designed to treat and/or prevent the programmed trend to obesity.

Resumen

Programación metabólica asociada a una restricción calórica gestacional leve en ratas y su potencial reversibilidad por la leptina

La prevalencia de la obesidad está incrementándose a nivel mundial y está emergiendo en los países en vías de desarrollo. La etiología de la obesidad es multifactorial, con interacciones complejas entre la base genética y factores sociales y ambientales, como el estilo de vida y los hábitos alimentarios. Además, las condiciones durante los primeros períodos de vida, particularmente la gestación y el período postnatal temprano, pueden programar el desarrollo de tejidos y órganos y causar efectos de por vida sobre las funciones metabólicas y fisiológicas, influyendo en el estado de salud. Considerando el interés en la prevención de la obesidad, el estudio de los efectos de la programación metabólica en las primeras etapas de la vida y su posible reversión son objetivos importantes de investigación. Nuestro grupo de investigación ha descrito anteriormente que la suplementación oral con dosis fisiológicas de leptina durante el período de lactancia en ratas tiene beneficios a corto y largo plazo sobre la salud metabólica, tales como una mayor protección contra la obesidad inducida por la dieta y sus trastornos metabólicos. En esta línea, estudios más recientes han demostrado que la suplementación con leptina durante la lactancia es capaz de revertir los efectos de la malprogramación sobre la estructura y función del hipotálamo y del tejido adiposo blanco observados a una edad juvenil en la descendencia de ratas sometidas a restricción calórica leve durante la gestación. Sin embargo, no se conocía todavía si los efectos beneficiosos de la leptina son duraderos y protegen frente al riesgo de alteraciones metabólicas relacionadas con la obesidad en la edad adulta.

En la presente tesis *doctoral* ***nos propusimos estudiar en ratas si las alteraciones metabólicas y el riesgo obesogénico en la edad adulta asociados a una restricción calórica leve (20%) durante la gestación pueden evitarse mediante la suplementación con leptina durante la lactancia. Por otra parte, también pretendimos utilizar este modelo animal para identificar posibles biomarcadores basados en transcritos en las células mononucleares de sangre periférica (PBMCs) en edad adulta, indicativos de un mayor riesgo de alteraciones metabólicas relacionados con la obesidad y sensibles a una intervención nutricional temprana.***

Los resultados de esta tesis doctoral muestran que la suplementación con dosis fisiológicas de leptina durante la lactancia previene el fenotipo dismetabólico en edad adulta asociado a una restricción calórica leve durante el embarazo y mejora las adaptaciones metabólicas a una dieta obesogénica. En edad adulta, la descendencia de ratas sometidas a una restricción calórica durante la gestación (CR) presentó una mayor adiposidad, junto con una mayor eficiencia energética, un menor gasto energético y una reducción de la actividad locomotora en comparación con los animales control, lo que sugiere un desequilibrio en la homeostasis energética. Además, los animales CR en edad adulta presentaron un índice HOMA-IR más elevado y unos niveles plasmáticos de leptina superiores que los controles. La mayoría de estas alteraciones se

normalizaron en animales CR suplementados con leptina oral durante el período de lactancia (CR-Leptina). Los animales CR también mostraron signos más severos de esteatosis hepática bajo una dieta occidental (*Western diet*) que los animales CR-Leptina, indicativos de una adaptación deteriorada a la dieta hipercalórica, y relacionados con la menor sensibilidad a la insulina. Por otra parte, los animales CR, pero no los CR-leptina, mostraron indicios de una menor capacidad de expansión del tejido adiposo blanco, junto con una capacidad lipogénica y lipolítica disminuida, también de acuerdo con la menor sensibilidad a la insulina y señalización de la leptina. La suplementación con leptina durante la lactancia también revirtió la disminución de la inervación simpática en el estómago sufrida por los animales CR, particularmente en edad juvenil, y restauró los niveles circulantes de grelina, que estaban disminuidos en los animales CR. Esto puede explicar la normalización de la relación leptina/grelina, considerado como un posible marcador clínico no invasivo de alteraciones metabólicas relacionadas con la obesidad.

Los modelos animales mencionados anteriormente (es decir, las ratas expuestas a una cierta desnutrición durante la gestación y las ratas tratadas con leptina durante la lactancia) se han utilizado además para identificar un conjunto de biomarcadores basados en transcritos en las PBMCs en edad adulta como posibles indicadores de predisposición a alteraciones relacionadas con el síndrome metabólico y sensibles a su reversión por una intervención nutricional en edad temprana. Entre los genes identificados, dos de ellos están relacionados con el control de la ingesta (*Agrp* y *Pomc*) y otros con el metabolismo lipídico, y con conexiones con la homeostasis de la insulina, esteatosis hepática y salud cardiovascular (*Pcyt2*, *Rxrb*, *Insig1*, *Npc1*, *Slc27a4*, *Apoo*, *Pla2g2a* y *Sort1*).

En conjunto, los resultados obtenidos en esta tesis aportan evidencias adicionales que apoyan la relevancia de la ingesta de cantidades apropiadas de leptina durante la lactancia para revertir las secuelas postnatales inducidas por una nutrición fetal deficiente, y por lo tanto podría ser considerada en la búsqueda de estrategias diseñadas para tratar y / o prevenir una tendencia programada a la obesidad.

Resum

Programació metabòlica associada a una restricció calòrica gestacional lleu en rates i la seva potencial reversibilitat per la leptina

La prevalença de l'obesitat està augmentant a tot el món i és emergent en els països en vies de desenvolupament. L'etiologia de l'obesitat és multifactorial, implicant interaccions complexes entre la base genètica i els factors socials i ambientals, com l'estil de vida i els hàbits alimentaris. A més, les condicions durant els primers períodes de la vida, sobretot la gestació i el període postnatal primerenc, poden programar el desenvolupament dels teixits i òrgans i causar efectes de per vida sobre les funcions metabòliques i fisiològiques, influint així en l'estat de salut. Tenint en compte l'interès en la prevenció de l'obesitat, l'estudi dels efectes de la programació metabòlica en etapes primerenques de la vida i la seva possible reversió, són objectius importants d'investigació. El nostre grup de recerca ha descrit prèviament que la suplementació amb dosis fisiològiques de leptina per via oral durant tot el període de lactància en rates té beneficis per a la salut metabòlica a curt i llarg terminis, tals com una major protecció contra l'obesitat induïda per la dieta i els seus trastorns metabòlics relacionats. En aquesta línia, estudis més recents han demostrat que la suplementació amb leptina durant la lactància és capaç de revertir els efectes d'una malprogramació sobre l'estructura i funció de l'hipotàlem i el teixit adipós blanc observats en edat juvenil a la descendència de rates sotmeses a una restricció lleu durant la gestació. No obstant això, no es coneixia encara si els efectes beneficiosos de la leptina eren de llarga durada, protegint enfront del risc d'alteracions metabòliques relacionades amb l'obesitat a l'edat adulta.

En la present tesi doctoral, ***ens vam proposar estudiar en rates si les alteracions metabòliques i el risc obesogènic a l'edat adulta associats a una restricció calòrica lleu (20%) durant la gestació es poden prevenir mitjançant l'administració de leptina durant el període d'alletament. D'altra banda, també vam pretendre utilitzar aquest model animal per identificar potencials biomarcadors basats en transcrits en cèl·lules mononuclears de sang perifèrica (PBMCs) a en l'edat adulta indicatius d'un major risc d'alteracions metabòliques relacionades amb l'obesitat i sensibles a la intervenció nutricional primerenca.***

Els resultats d'aquesta tesi doctoral mostren que la suplementació amb dosis fisiològiques de leptina durant la lactància evita el fenotip dismetabòlic a l'edat adulta associat a una restricció calòrica lleu durant l'embaràs i millora les adaptacions metabòliques a una dieta obesogènica. A l'edat adulta, la descendència de rates sotmeses a una restricció calòrica durant la gestació (CR) va presentar un augment de l'adipositat, juntament amb una major eficiència energètica, una menor despesa energètica, i una reducció de l'activitat locomotora en comparació amb els animals control, la qual cosa suggereix un desequilibri en la homeòstasi energètica. D'altra banda, els animals CR adults mostraren un major índex HOMA-IR i uns nivells de leptina en plasma més elevats que els controls. La majoria d'aquestes alteracions es van normalitzar en els

animals CR suplementats amb leptina per via oral durant el període d'alletament (CR-leptina). Els animals CR també van mostrar signes més greus d'esteatosi hepàtica sota una dieta occidental (*Western diet*) que els animals CR-leptina, indicatius d'una mala adaptació a la dieta hipercalòrica i relacionats amb la menor sensibilitat a la insulina. A més, els animals CR, però no els animals CR-leptina, van mostrar indicadors d'una menor capacitat d'expansió del teixit adipós blanc, juntament amb una menor capacitat lipogènica i lipolítica, també d'acord amb la menor sensibilitat a la insulina i senyalització de la leptina. També es mostra que la suplementació amb leptina durant la lactància reverteix la menor innervació simpàtica a l'estómac que pateixen els animals CR, especialment a una edat juvenil, i també restaura els nivells de grelina circulants, que estaven disminuïts en els animals CR. Això pot explicar la normalització de la relació leptina/grelina, que es considera com un posible marcador clínic no invasiu d'alteracions metabòliques relacionades amb l'obesitat.

Els models animals esmentats anteriorment (és a dir, les rates exposades a una certa desnutrició durant la gestació i rates tractades amb leptina durant la lactància) s'han utilitzat per identificar un conjunt de biomarcadors basats en transcrits en PBMCs a l'edat adulta com a indicadors potencials de predisposició a alteracions relacionades amb la síndrome metabòlica i sensibles a la seva reversió per una intervenció nutricional en edats primerenques. Cal destacar que entre els gens identificats, dos d'ells estan relacionats amb el control de la ingesta (*Agrp* i *Pomc*), i altres amb el metabolisme lipídic, i amb connexions a l'homeòstasi de la insulina, l'esteatosi hepàtica, i la salut cardiovascular (*Pcyt2*, *Rxrb*, *Insig1*, *Npc1*, *Slc27a4*, *Apoo*, *Pla2g2a* i *Sort1*).

En conjunt, els resultats obtinguts en aquesta tesi aporten proves addicionals que recolzen la rellevància de la ingesta de quantitats apropiades de leptina durant la lactància per revertir les seqüeles post-natals induïdes per una nutrició fetal deficient, i que per tant podria ser considerada en la recerca d'estratègies dissenyades per tractar i / o prevenir una tendència programada a l'obesitat.

List of Original Articles

This thesis is based on 3 original manuscripts (numbers 1- 3, listed below):

1. **Szostaczuk N.**, Priego T., Palou M., Palou A., Picó C. **Oral leptin supplementation throughout lactation in rats prevents later metabolic alterations caused by gestational calorie restriction.** *Int J Obes (Lond),* 2017; 41(3):360-371. doi: 10.1038/ijo.2016.241

2. **Szostaczuk N.**, Konieczna J., Sánchez J., Palou A., Picó C. **Leptin intake at physiological doses throughout lactation in rats restores the altered stomach sympathetic drive caused by mild gestational calorie restriction.** Manuscript to be submitted for publication

3. **Szostaczuk N.**, van Schothorst E. M., Sánchez J., Priego T., Bekkenkamp-Grovenstein M., Keijer J., Palou A., Picó C. **Identification of blood cell transcriptome-based biomarkers in adulthood predictive of increased risk to develop metabolic disorders due to gestational undernutrition and sensitive to its reversion by leptin supplementation.** Manuscript to be submitted for publication

My contribution to each manuscript was as follows:

Manuscript 1: I carried out most of the animal experiment and performed all the analysis included in the manuscript. I also analyzed all data obtained, participated in the discussion and I have wrote a first version of the manuscript.

Manuscript 2: I performed the animal experiment in adult age. I have collaborated in the analysis of circulating parameters, RNA extraction and gene expression analysis, and Western blot analysis of stomach. I have also collaborated in the analysis of the data, discussion of the results and wrote a first version of the manuscript.

Manuscript 3: I carried out the animal experiment. I performed the isolation of PBMCs from blood samples. I performed the RNA extraction and, with the collaboration of other authors, I have participated in the performance of the microarray analysis and data analysis. I have also participated in the discussion of the results and I wrote a first version of the manuscript.

1. GENERAL INTRODUCTION

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1.1 OBESITY

Obesity is a complex, non-communicable, and largely preventable disease, which is gaining increasing importance on a global scale and is emerging in developing countries (Malik et al., 2013b). The fundamental law of the energetics of obesity is governed by the balance between energy intake and energy expenditure. Thus, it is widely agreed that obesity results from a prolonged imbalance between energy intake and energy expenditure (Hill et al., 2012).

Obesity can be classified using the Body Max Index (BMI), which is calculated as weight in kilograms divided by the square of the height in meters (Keys et al., 1972). The greater the value of the BMI, the greater is the health risk. The latest publication of the World Health Organization (WHO) in relation to obesity indicates that in 2014, worldwide, more than 1.9 billion adults were overweight (BMI in $kg/m^2 \geq 25$); of these, over 600 million adults were obese (BMI in $kg/m^2 \geq 30$) (WHO, 2016). Overall, about 13% of the world's adult population (11% of men and 15% of women) was obese and 39% of adults aged 18 years and over (38% of men and 40% of women) were overweight. The worldwide prevalence of obesity increased more than doubled between 1980 and 2014, and overweight and obesity are linked to more deaths worldwide than underweight since it is a key risk factor in the development of insulin resistance, type 2 diabetes, hypertension, and cardiovascular diseases (WHO, 2016).

The etiology of obesity is multifactorial, involving complex interactions among the genetic background, hormones, and different social and environmental factors, such as sedentary lifestyle and unhealthy dietary habits (Ogden et al., 2007). Moreover, growing evidence shows that conditions during early critical periods of life, such as gestation and the immediate postnatal period, may program the development of tissues and organs and can elicit lifelong effects on metabolic and physiological functions and influence health status (Barker, 1995; Godfrey and Barker, 2000; Patel and Srinivasan, 2002). This has been conceptualized as the developmental programming hypothesis (Barker, 1998; Langley-Evans et al., 1996), which derives from human epidemiological studies and intervention studies in animal models, and provides the link between periconceptional, fetal, and infancy periods and the subsequent development of metabolic disorders in later life. In this context, it is well established that alterations in the nutritional environment of early life can lead to alterations in energy balance regulatory systems and favor the development of obesity and related metabolic complications (Reynolds et al., 2015). Therefore, the development of strategies for optimizing early life nutrition as well as to reverse potential adverse consequences of developmental malprogramming during critical windows of intervention are critical for attaining lifelong improvements in health.

1.1.1. Energetics of obesity and energy balance regulation

Obesity is the result of a chronic energy imbalance caused by either excessive energy intake or insufficient energy expenditure. In turn, low energy expenditure may be due to low resting metabolic rate, insufficient energy expenditure through physical activity, low diet-induced thermogenesis, or a combination of all these components, which may contribute towards positive energy balance and subsequent weight gain (Roberts, 1995). Besides energy intake and expenditure, other determinants of energy balance, such as adipogenesis, i.e. the regulation of cell growth and differentiation giving rise to mature adipocytes, may also play an important role due to its function in processing the largest energy reserve as triacylglycerides (TG) in the body (Palou et al., 2000). The process of adipocyte differentiation is highly regulated by expression of diverse genes that lead to each adipocyte phenotype characteristic of each stage of differentiation. Thus, factors that regulate the number of adipocytes and their maturity and differentiation should be taken into account when considering possible causes of obesity.

The regulation of energy balance is a dynamic process in which changes in one component may elicit biological and/or behavioral "compensation" in other components of the system. These self-regulating or compensatory responses act with the aim of minimizing disturbances in energy homeostasis, which in turn are reflected in the regulation of body weight (Hopkins and Blundell, 2016; King et al., 2007). The nature and extent of compensation of energy deficit or surfeit will play an important role in determining an individual´s susceptibility or resistance to weight loss (Byrne et al., 2012). The notion of biological and behavioral compensation highlights the fact that the regulation of energy balance, and the mechanisms that drive energy intake and expenditure, are tremendously complex (Hopkins and Blundell, 2016). The physiological regulation of energy balance involves the complex interaction between central regulatory pathways and multiple peripheral feedback signals arising from adipose tissue and the gastrointestinal tract, principally, among other organs, and these biological regulatory mechanisms also interact with environmental and psychosocial factors in the overall expression of body weight (Berthoud, 2006).

A complex system has evolved which allows the brain to read, interpret and integrate a wide range of signals and to elicit appropriate changes in food intake and energy expenditure as a result of the information received from both short and long-term central and peripheral signals. A series of short-term signals derived from the gastrointestinal tract govern meal size, together with insulin and leptin and circulating nutrients which offer long-term regulation. These signals act at a variety of central nervous system sites, but the majority of pathways converge at the hypothalamus, which produces numerous peptides and neurotransmitters that influence feeding and energy expenditure (Lenard and Berthoud, 2008).

1.1.2. Main tissues involved in the maintenance of energy homeostasis

1.1.2.1. Hypothalamus

Many studies conducted in the 1950s and 1960s highlighted the great importance of the hypothalamus in regulating food intake (Anand and Brobeck, 1951; Anand et al., 1964; Andersson et al., 1961). Since then, large experimental evidence in understanding the neurobiology of obesity has strongly established that the mediobasal hypothalamus is a fundamental nexus in the neuronal hierarchy controlling whole-body energy balance (Schneeberger et al., 2014). The hypothalamus, located in the mediobasal par of the brain, encompasses several anatomically well-defined nuclei including the arcuate nucleus (ARC), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), lateral hypothalamic area (LHA), and paraventricular nucleus (PVN) (Schneeberger et al., 2014) (see Figure 1.1). Within each nuclei, neuronal populations are characterized by their expression of neuropeptides, which are involved in the control of food intake and energy balance. Lesions in the VMN are associated with hyperphagia and obesity (Stellar, 1954), while lesions in the LHA cause hypophagia and a decrease in body weight (Anand and Brobeck, 1951). Within the basal part of the hypothalamus, the ARC controls long-term food intake throughout two neuronal populations, each characterized by the expression of specific neuropeptides. On the one hand, pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) neurons have potent effects on energy homeostasis, providing a strong anorexigenic effect by decreasing food intake and body weight. On the other hand, agouti-related peptide (AgRP) and neuropeptide Y (NPY) neurons are the second population of neurons that have a potent orexigenic effect (Dietrich and Horvath, 2009). Accordingly, in a situation of negative energy balance, such as the fasting state, the expression of AgRP and NPY is increased and that of POMC and CART decreases. Conversely, during a state of energy excess, expresion levels of orexigenic peptides are diminished while those of anorexigenic factors are elevated. It is well established that both neuropeptides are, in addition, important regulators of peripheral metabolism, as has been reported by the importance of intact AgRP signalling in adult mice to maintain normal lipid and glucose homeostasis in peripheral tissues, such as the liver, muscles and the pancreas (Varela and Horvath, 2012).

Many observations suggest that the VMN also plays an important role in processing and integrating satiety and adiposity signals (Chao et al., 2012; Dhillon et al., 2006; Kim et al., 2011; Majdic et al., 2002). Medial VMN neurons project to the ARC and provide excitatory inputs onto POMC neurons (Sternson et al., 2005). VMN neurons express anorexigenic brain-derived neurotrophic factor (BDNF) (Xu et al., 2003), which stimulates the expression of anorexigenic melanocortin 4 receptor (MC4R) (Cordeira and Rios, 2011).

The DMN contains orexigenic NPY neurons which project to the PVN, LH/perifornical area, and to the anteroventral peroventricular nucleus (Guan et al., 1998). Furthermore, NPY expression in the DMN increases under hyperphagic conditions (e.g lactation or diet-induced obesity) (Bi et al., 2003).

The LHA is considered as a feeding center and serves as an interface that connects the homeostatic circuitry to the hedonic circuitry. LHA neurons express orexin, an orexigenic neuropeptide present in two isoforms (A and B) (Sakurai et al., 1998) and melanin-concentrating hormone (MCH), also with an orexigenic function (Bittencourt et al., 1992).

The PVN contains anorexigenic neurons required for the maintenance of nomal energy balance and body weight. The PVN receives projections from many others hypothalamic areas. AgRP neurons in the ARC innervate PVN neurons through inhibitory GABAergic inputs (Atasoy et al., 2012), and POMC neurons in the ARC also project to the PVN and counteract AgRP neuron action (Cone, 2005). The PVN is an important anatomical route of the hypothalamus that connects the forebrain to the hindbrain.

Neurons in the mediobasally ARC receive information regarding the status of the energy reserves through peripheral hormones, such as leptin and insulin, that circulate in amounts related to body fat stores. Then the information is integrated and passed on to other hypothalamic nuclei, such as the PVN and the LHA, and from there on to output functions. The process is also influenced by satiety hormones originated from the gastrointestinal tract (Schwartz et al., 2000).

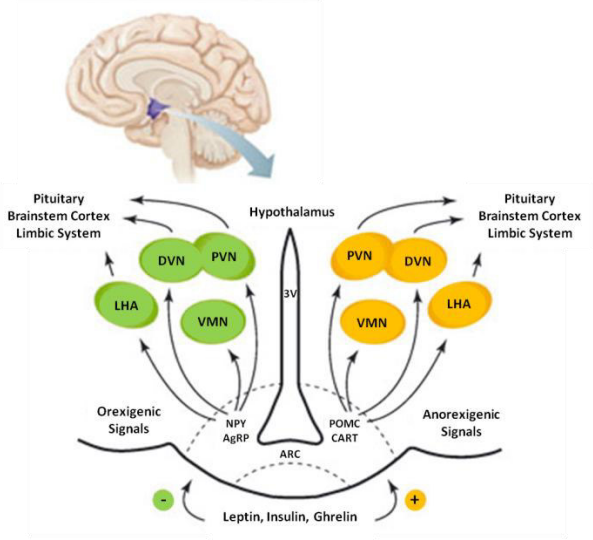

Figure 1.1. Hypothalamic nuclei involved in feeding control. Orexigenic signals that decrease feeding are shown to the left and anorexigenic signals that stimulate feeding are shown to the right of the third ventricle (3V). The lateral hypothalamic area (LHA), ventromedial nucleus (VMN), dorsomedial nucleus (DMN) and paraventricular nucleus (PVN) all project within and outside the hypothalamus to modulate activity. Abbreviations: AgRP, agouti-related protein; ARC, arcuate nucleus; CART, cocaine- and amphetamine-regulated transcript; NPY, neuropeptide Y and POMC, pro-opiomelanocortin. Adapted from (Crowley et al., 2002).

1.1.2.2. White adipose tissue

Adipose tissue is a type of loose connective tissue constituted of lipid-filled cells, known as adipocytes, which is enclosed by a matrix of blood vessels, collagen fibers, fibroblast and immune cells. In humans, white adipose tissue (WAT) is the principal form of adipose tissue, and is characterized by adipocytes with a single lipid droplet and an eccentric nucleus. Besides being the site where energy is stored in the form of TG, it is well described that WAT plays an active role in energy homeostasis as well as the control of neuroendocrine, autonomic and immune functions (Ahima and Flier, 2000a). Adipose tissue metabolism is dynamic, in which the supply and removal of substrates in

the blood is highly regulated according to the nutritional status. This organ also possesses the ability to modulate its own metabolic activities, including differentiation of new adipocytes and production of blood vessels as necessary to shelter increasing fat stores. In addition, WAT, through adipocyte signals to other tissues, is able to regulate their energy metabolism in accordance with the nutritional state of the body (Frayn et al., 2003).

Lipogenesis and lipolysis are the main metabolic processes attributed to WAT (see Figure 1.2). Some of the fatty acids used for the synthesis and storage of TG may be synthesized from carbohydrates through the pathway of *de novo* lipogenesis, although this contribution is generally insignificant in humans except in situations of massive and prolonged carbohydrate overfeeding, where insulin promotes the synthesis of lipids (Solinas et al., 2015). In adipocytes, glucose is stored primarily as lipid, owing to an increased uptake of glucose through glucose transporter type 4 (GLUT4) and the activation of lipid synthetic enzymes, including pyruvate dehydrogenase (PDH), fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC). Insulin also inhibits lipolysis in adipocytes, primarily through the inhibition of the enzyme hormone sensitive lipase (HSL) (Anthonsen et al., 1998). Most of the TG deposited in adipose tissue arises from the pathway mediated by the enzymatic activity of lipoprotein lipase (LPL), which is responsible of TG hydrolysis from circulating TG-rich lipoproteins (chylomicrons and very low density lipoproteins) followed by uptake of the resulting non-esterified fatty acids (NEFA) into cells and esterification to glicerol 3-phosphate. Both these processes are stimulated by insulin in the postprandial period (Frayn et al., 2003). Consecutively, the stored energy is released from WAT to meet the energy needs of other organs. The sequential hydrolysis of TG in adipocytes producing NEFA is catalyzed by a cascade of lipolytic enzymes, with different substrate preferences (Watt and Steinberg, 2008). The committed enzyme catalyzing the first step of TG hydrolysis is adipose trigliceride lipase (ATGL) (officially annotated as Patatin Like Phospholipase Domain Containing 2 (PNPLA2)), converting TG to diacylglycerol (DG); HSL is mainly responsible for the hydrolysis of DG to monoacylglycerol (MG) and MG lipase (MGL) hydrolyzes MG. Free fatty acids are transported to the plasma membrane bound to adipocyte fatty acid-binding protein (aP2, also known as FABP4) and are then transported across the plasma membrane into the circulation by one of several fatty acid transport proteins. The glycerol released through the action of monoacylglycerol lipase (MGL) is transported across the plasma membrane via the action of aquaporin 7 (AQP7) (Lebeck, 2014). In adipose tissue, ATGL and HSL are responsible for more than 90% of TG hydrolysis (Schweiger et al., 2006).

Lipolysis is regulated by complex regulatory mechanisms involving lipase expression and activity, as well as lipid droplet-associated proteins. The first two steps, catalyzed by ATGL and HSL, are considered to be the regulatory steps in lipolysis. Since ATGL and HSL hydrolyze TGs in a coordinated manner, it is expected that they share many regulatory similarities. However, the mechanisms of enzyme regulation differ markedly between the two lipases. Lipolysis is regulated by catecholamines (epinephrine/norepinephrine) released from the SNS and by glucagon. Binding of

epinephrine/norepinephrine and glucagon to their respective receptors (β3-adrenergic and glucagon receptors, respectively) triggers activation of adenylate cylcase (AC). When intracellular levels of cAMP increase, they activate protein kinase A (PKA), with the consequent phosphorylation and activation of both perilipin-1 and HSL. Phosphorylation of HSL determines the consequent translocation of the enzyme to the lipid droplet surface (Egan et al., 1992), inducing a conformational change that increases the exposed hydrophobic surface area to facilitate its binding to the lipid droplet (Krintel et al., 2009). These events lead to increased lypolisis. The phosphorylation of perilipin-1 leads to the release of the ATGL co-activator, ABHD5 (also known as gene identification-58 (CGI-58)) (Lass et al., 2006), the gene/protein responsible of stimulating the enzymatic activity of ATGL to hydrolyse stored TG. ATGL phosphorylation is also necessary to start with the lipolitic cascade in adipocytes together with the interaction of its co-activator (Pagnon et al., 2012). Unlike HSL, ATGL phosphorylation is not PKA-dependent. It has been shown that AMPK phosphorylates ATGL which results in increased lipase activity (Ahmadian et al., 2011; Kim et al., 2016); however, the regulation of ATGL and the role of AMPK still remains unclear.

The actions of insulin, which counter the effects of catecholamines or glucagon, are primarily the result of the activation of PKB/AKT which then phosphorylates and activates phosphodiesterase (PDE) leading to a reduced level of cAMP and reduced activity of PKA. The inhibiton of lipolysis mediated by insulin is also associated with transcriptional downregulation of ATGL and HSL expression (Kershaw et al., 2006; Kralisch et al., 2005).

Besides regulation of ATGL activity, this enzyme is regulated at the transcriptional level. ATGL expression is induced by glucocorticoids, which are peroxisome proliferator-activated receptor (PPAR) agonists, and its expression is increased by fasting and decreased by refeeding. Thus, unlike HSL, which acutely affects lipolysis, primarily via posttranslational mechanisms, ATGL may mediate more long-term effects on lipolysis via transcriptional mechanisms (Kershaw et al., 2006). The expression profile of HSL, within adipocytes, essentially mirrors that of ATGL (Zechner et al., 2012).

Considering the above described functions of WAT regulating fat stores, it may represent the largest endocrine tissue of humans. Its pleiotropic nature is based on the ability of fat cells to secrete numerous hormones, growth factors, enzymes, cytokines, complement factors and matrix proteins. WAT also expresses receptors for most of these factors that are implicated in the regulation of many processes including food intake, energy expenditure, metabolism homeostasis, immunity and blood pressure homeostasis (Costa and Duarte, 2006; Matsuzawa, 2006). Substances such as adiponectin, leptin, angiotensin, resistin, visfatin, acylation stimulating protein (ASP), sex steroids, glucocorticoids, tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), and NEFA, among others, are secreted by WAT. Unbalanced production of pro- and anti-inflammatory adipocytokines in obese adipose tissue may contribute to many aspects of the metabolic syndrome (MetS). Oversecretion of potentially harmful adipocytokines,

such as plasminogen activator inhibitor-1(PAI-1), TNFα or visfatin, and hyposecretion of potentially beneficial adipocytokines, such as adiponectin, might be major mechanisms involved in lifestyle-related diseases, including diabetes mellitus, hyperlipidemia, hypertension and atherosclerosis, comprising the so called MetS (Seneff et al., 2011; Stepien et al., 2012). Regarding leptin, this hormone is the afferent signal in a negative feedback loop that maintains homeostatic control of WAT. It circulates in the blood and acts on the brain to regulate food intake. When fat mass falls, plasma leptin concentrations fall too, stimulating appetite and suppressing energy expenditure until fat mass is restored. When fat mass increases, leptin levels increase, suppressing appetite until excess weight is lost (Friedman, 2011). This system maintains homeostatic control of adipose tissue mass (see section 1.1.3).

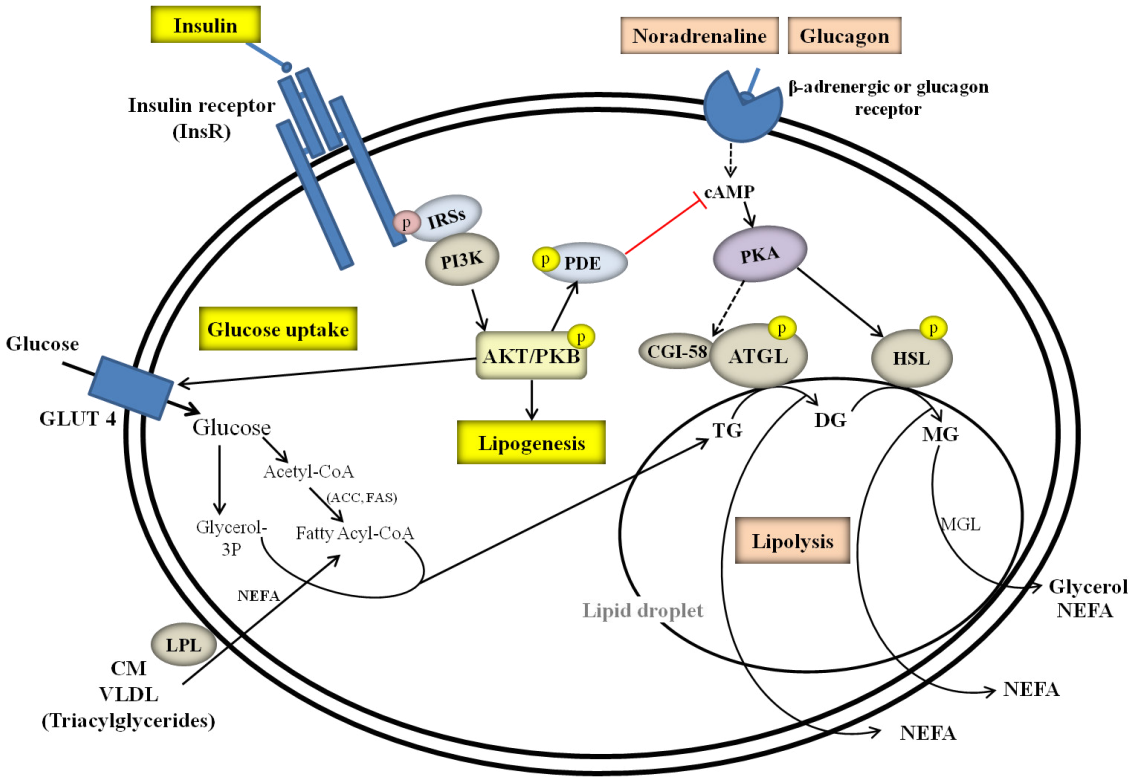

Figure 1.2. Intracellular pathways and factors that regulate lipogenesis and lipolysis in the adipocyte. TG circulate in blood in the form of lipoproteins (CM and VLDL), and NEFA that are released from these lipoproteins and catalyzed by LPL, diffuse into the adipocyte. Intracellular NEFA are then converted into fatty acyl-CoA and reesterified to form TG using glycerol-3P, generated from glucose metabolism. NEFA may also originate from acetyl-CoA (de novo lipogenesis) by the action of lipogenic enzymes ACC and FAS. Insulin binding to InsR induces receptor tyrosine autophosphorylation and activation of IRS, PI3K, and Akt/PKB signaling pathways. Insulin enhances the storage of fat as TG by increasing LPL and lipogenic enzyme activities. It also facilitates the transport of glucose by stimulating GLUT4 glucose transporter. Moreover, phosphorylation and activation of PDE are key events in the antilipolytic action of insulin, decreasing cAMP level in adipocytes. Noradrenaline and glucagon (each released during different metabolic states) bind β-adrenergic or glucagon receptors as appropiate and activate lipolysis, during which intracellular levels of cAMP increase leading to PKA activation with the consequent phosphorylation and activation of HSL. The release of the ATGL co-activator, CGI-58, is responsible of stimulating the enzymatic activity of ATGL to hydrolyse the stored TG in DG, which are substrates for the PKA-activated HSL. HSL hydrolyzes DG to MG, which are finally hydrolyzed by the action of monoacylglycerol lipase (MGL). The resulting NEFA and glycerol are released outside of the adipocyte. Abbreviations: CM: chylomicrons; VLDL: very-low-density lipoprotein; LPL: lipoprotein lipase; NEFA: non-esterified free fatty acids; glycerol-3P: glycerol 3-phosphate; ACC: acetyl-CoA carboxylase; FAS: fatty acid synthase; InsR: insulin receptot; IRS: insulin receptor substrate; PI3K phosphatidylinositol 3-kinase; Akt/PKB: protein kinase B; PDE nucleotide phosphodiesterase; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; ATGL: adipose triacylglyceride lipase; CGI-58: gene identification-58; HSL: hormone-sensitive lipase; TG: triacylglyceride; DG: diacylglicerol; MG: monoacylgliceride.

1.1.2.3. Brown adipose tissue

Brown adipose tissue (BAT) plays an important role in energy homeostasis and is the main site of adaptive thermogenesis in animals. The presence and function of BAT in adult humans has been recognized in the last decade, and this has generated a great interest for this tissue as a target for obesity therapy (Nedergaard et al., 2007). The human BAT depots are differently located from those in rodents. The main deposits have been found in the supraclavicular and in the neck regions with some additional paravertebral, mediastinal, para-aortic and suprarenal localization (Nedergaard et al., 2007). Brown adipocytes are characterized by multilocular lipid droplets and a greater amount of mitochondria that contain uncoupling protein 1 (UCP1) in their inner membrane, which is the molecular base for thermogenesis (Cinti, 2005; Sell et al., 2004). 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 (Cannon and Nedergaard, 2004). The sympathetic innervation of BAT is the most significant physiological effector of the thermogenic process, where the terminals of sympathetic nerves present in BAT are responsible for the release of norepinephrine (NE), that turn on a cascade of intracellular events ending in activation of UCP1, together with other processes, including TG mobilization, thus providing the fatty acids necessary for UCP1 activation (Cannon and Nedergaard, 2004; Palou et al., 1998). Nuclear receptors and cofactors, such as peroxisome proliferator-activated receptor gamma (PPARγ) and its coactivator, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1A), regulate *Ucp1* gene expression (Lowell and Spiegelman, 2000). Furthermore, the expression of *Ucp1* is modulated by diet and also metabolic hormones such as leptin and glucocorticoids (Sell et al., 2004). Leptin acts at a central level to decrease food intake and to increase thermogenesis (Commins et al., 1999), and glucocorticoids are down-regulators of *Ucp1* gene expression in BAT (Arvaniti et al., 1998). Thyroid hormones also play a key role in the activation of adaptive thermogenesis. 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 2 (DIO2) (Martinez de Mena et al., 2010), a selenoenzyme that locally produces T3, via 5' deiodination of thyroxine (T4). It has been described that clusters of UCP-1 expressing adipocytes with thermogenic capacity are also developing in WAT in response to diverse stimulus such as cold exposure (Vitali et al., 2012). The brown adipocytes appearing in WAT have been called inducible "brite" (brown in white) or beige adipocytes, and may also involve transdifferentiation processes of white-to-brown adipose cells (Giralt and Villarroya, 2013; Harms and Seale, 2013).

Physiologically, the hypothalamic neural system integrates central and peripheral signals of energy status to regulate both food intake and energy expenditure to maintain energy homeostasis. Thus, this enormous effect on energy utilization highlights BAT as an appealing target for the prevention and treatment of obesity and other metabolic disorders in humans (Cypess et al., 2013; Timmons and Pedersen, 2009).

1.1.2.4. Liver

The liver is an organ with a pivotal role in body energy metabolism. It is responsible for a spectrum of functions, including the uptake, metabolism, conjugation, and excretion of various endogenous and foreign substances, in which transporters play an important role. The liver also provides an immunological function, as its reticuloendothelial capacity plays a role in phagocytosis, and participates in the clearance of/is in charge of clearing microorganisms and endotoxins from portal blood (Schindl et al., 2006). Liver acts as a hub to metabolically connect various tissues, including skeletal muscle and adipose tissue. Liver energy metabolism is tightly controlled, where multiple nutrient, hormonal, and neuronal signals have been identified to regulate glucose, lipid, and amino acid metabolism.

Hepatocytes, the main cell type in the liver (~80%), are the main responsible for glucose synthesis. Blood glucose enters hepatocytes via GLUT2, a plasma membrane glucose transporter (Seyer et al., 2013). Glucose is phosphorylated by glucokinase (GK), the major glucose-phosphorilating enzyme in hepatocytes, to generate glucose 6-phosphate (G6P). As G6P is unable to be transported by glucose transporters, it is retained within hepatocytes, and in the fed state, it acts as a precursor for glycogen synthesis. Insulin, secreted by pancreatic β cells, stimulates glycogen synthase by activating AKT which phosphorylates and inactivates glycogen synthase kinase 3 (GSK-3), thus increasing glycogen synthesis. In the fasted state, G6P is transported into the endoplasmic reticulum (ER) and dephosphorylated by glucose-6-phosphatase (G6Pase) to release glucose. The fall of portal venous concentration of glucose and secretion of glucagon act as a signal to initiate liver glycogen metabolism characteristic of the fasted or postabsorptive state (Shikama et al., 1980).

Hepatocytes have great flexibility in selecting metabolic fuels (glucose and/or fatty acids). Fuel selection is regulated by both nutrient and hormonal signals. Glycolysis is dominant in the fed state in which glucose is abundant. Glycolytic intermediates and products are used to synthesize lipids, amino acids, and other important molecules in addition to be completely oxidized to generate ATP. TG are syntethised from fatty acids and glycerol-3-phosphate during the fed state and, together with cholesterol esters, they are either stored in lipid droplets in hepatocytes or secreted into circulation packed into very low-density lipoprotein (VLDL) particles and delivered to adipose tissue and other extrahepatic tissues through the bloodstream (Rui, 2014).

In liver, insulin and glucagon act in a cooperative way to control the fasted-to-fed state transition by promoting gene expression of those sets of genes involved in each metabolic pathway. The *Gk* gene is regulated by insulin and glucagon at the transcriptional level, and post-translationally by the GK regulatory protein (GKRP). The importance of GKRP lies in its ability to extend the half-life of GK,which allows the release of GK from a nuclear pool that is transported into the cytoplasm after a meal, ensuring that the signal afforded by glucose metabolism will be present to help in the promoting of the fasting-to-fed transition of the hepatocyte (Collier and Scott, 2004; Slosberg et al., 2001).

During short-term fasting periods, the liver produces and releases glucose mainly through glycogenolysis. During prolonged fasting, in which glucose levels are low, hepatocytes switch to fatty acid β oxidation for energy supply. Therefore, stored fatty acids are released from the adipocytes and translocated to the liver from the bloodstream. Once inside the cell, long-chain fatty acids are esterified to acyl-CoA. They enter into the mitochondria by the action of carnitine palmitoyltransferase 1 (CPT1) that catalyzes the formation of acylcarnitine from acyl-CoA, allowing the fatty acid moiety to be transported across the inner mitochondrial membrane via carnitine translocase, which exchanges long-chain acylcarnitines for carnitine. The conversion of acyl-CoA to acylcarnitine by CPT1 is the rate limiting step of β-oxidation. The inner mitochondrial membrane CPT2 converts acylcarnitine back to acyl-CoA. Inside the mitochondrial matrix, fatty acids are oxidized through the β-oxidation pathway to produce energy. This occurs by cleaving two carbons every cycle to form acetyl-CoA. This process continues until the entire chain is cleaved into acetyl-CoA units. The final cycle produces two separated acetyl-CoAs. Under normal conditions, acetyl-CoA is further oxidized by the mitochondrial tricarboxylic acid (TCA) cycle. The NADH and FADH2 produced by both fatty acid β-oxidation and the TCA cycle are used by the electron transport chain to produce ATP. However, if the amounts of acetyl-CoA generated in fatty-acid β-oxidation challenge the processing capacity of the TCA cycle, as occurs under fasting conditions, but also by vigorous exercise, high-fat diets or other conditions, acetyl-CoA is then used instead in biosynthesis of ketone bodies and are therefore a source of energy for other tissues, such as the brain, which cannot directly metabolize fatty acids.

Peroxisome proliferator-activated receptor alpha (PPARα) is the master regulator of fatty acid β oxidation and promotes fatty acid β oxidation in both the mitochondria and peroxisomes (Kersten et al., 1999). PPARα expression in the liver is higher in the fasted state, and it regulates genes involved in hepatic fatty acid uptake, such as the gene encoding for the fatty acid transport protein (*Fatp*) and the fatty acid binding protein (*Fabp*). PPARα activation results in a reduction of plasma TG levels, achived on one hand by the induction of genes that decrease the availability of TG for hepatic VLDL secretion, and on the other by the induction of genes that promote lipoprotein lipase-mediated lypolisis of TG-rich plasma proteins (Duval et al., 2007).

1.1.2.5. Stomach

The stomach, located between the esophagous and the small intestine, carries out several functions, such as storage, dissolution and partial digestion of macromolecules present in food, along with the regulation of the rate at which the contents of the stomach empty into the small intestine (Eric Widmaier, 2014). The reservoir capacity of the stomach allows it to increase its volume significantly while internal pressure increases only slightly. The stimulation of mechanoreceptors in the mouth and pharynx induces vago-vagal reflexes which cause a relaxation of the gastric reservoir to prepare the stomach to receive the food bolus. Anatomically, the gastric reservoir is predominantly located in the fundus of the stomach (O'Connor and O'Morain, 2014). In

addition to the reservoir function, the stomach also plays an important motility role as a pump, which anatomically is provided by the distal two thirds of the corpus, the antrum and the pylorus.

Stomach acid secretion is a very important non-immunological defence against invading pathogens as well as being an important mechanism for vertebrates to have more complex diets. Stimulation of acid secretion involves the translocation of H^+/K^+-ATPases to the apical membrane of the parietal cell. The regulation of acid secretion is a complex milieu of neurological, endocrine and paracrine factors to maintain an optimal acid output (El-Omar et al., 1997). Pepsinogen is a precursor for pepsin, a digestive enzyme specifically produced in the gastric mucosa. Two isozymogens are expressed in the human stomach, PGI and PGII, with different biochemical and immunological properties (Samloff, 1971). The major digestive function of pepsinogen is to cleave proteins into polypeptides. The release of both pepsinogen and HCl from the stomach lining is controlled by the presence of gastrin and vagal activity.

The stomach is also an important endocrine organ producing an array of peptide hormones important for both enteric and non-enteric physiology, including ghrelin (Kojima et al., 1999) and leptin (Bado et al., 1998; Cinti et al., 2001; Oliver et al., 2002). Both endocrine and paracrine mediators exert control over the maintenance of gastric secretory functions. Leptin production was first described in rats (Bado et al., 1998), and later in humans (Cinti et al., 2001). It was localized in the lower half of the fundic glands in both the granules of chief cells and of an specific endocrine cell type, the P cells, suggesting both exocrine and endocrine leptin signaling action (Cinti et al., 2001). Gastric leptin is secreted by the stomach in response to food intake (Cinti et al., 2000) and to peptide hormones, such as CCK, pentagastrin and secretin (Bado et al., 1998; Sobhani et al., 2000), suggesting that gastric leptin has a physiologycal role in the short-term response to food intake (Pico et al., 2002). In the stomach, leptin deficiency increases the rate of gastric emptying, increases transit activity in the jejunum, and shortens total transit time in the small intestine (Kiely et al., 2005). In the preprandial state, leptin produced by gastric cells appears to decrease the absorption of glucose, whereas in the postprandial state, leptin has an important role in increasing the uptake of glucose (Molina et al., 2000).

Ghrelin on the other hand is a peptide hormone with a hydrophobic octanoyl moiety esterified to the third residue, serine. Ghrelin was first isolated from the rat stomach in 1999 by Kojima and collegues and described as the endogenous ligand for the growth hormone secretagogue receptor (GHSR) (Kojima et al., 1999). In addition to its ability to stimulate GH secretion and gastric motility, the most important role of ghrelin seems to be the stimulation of appetite and the regulation of energy homeostasis by the induction of a positive energy balance leading to body weight gain (Inui et al., 2004).

Finally, another hormone of interest secreted by the stomach is gastrin. This is a peptide hormone that stimulates secretion of gastric acid (HCl) by the parietal cells of the stomach and aids gastric motility. Gastrin is released from the G cells of the antral mucosa and travels through the bloodstream to the corpus where the enterochromaffin-

like cells are stimulated to secrete histamine which, in turn, stimulates the parietal cells to secrete acid. (Payne and Gerber, 1992). Gastrin also stimulates mucosal development and growth within the stomach.

1.1.3. Main hormones involved in the regulation of the energy homeostasis

1.1.3.1. Leptin and insulin signaling pathways

Leptin hormone is one of the main adipokines secreted by the WAT in a positively-related manner to the total amount of body fat content. It reaches the CNS through a saturable transport system and conveys information about the energy status of the organism (Margetic et al., 2002). In turn, insulin is secreted by the pancreatic β-cells, an action which depends on the level of blood glucose in the short term, and on the level of adiposity in the long term (Margetic et al., 2002).

Leptin and insulin act centrally and periferically, and their actions are mediated by the long form leptin receptor (ObRb) and the insulin receptor (InsR), respectively. Their main central action are the overall control of energy homeostasis, particularly feeding behavior, exhibiting an anorexigenic effect, and increasing energy expenditure (Woods and Seeley, 2000). Both the ObRb and InsR are expressed in diverse areas of the CNS, including the hypothalamus. In the ARC, they are highly present in the AgRP and POMC neurons, which are direct targets of leptin and insulin (Cowley et al., 2001; Cheung et al., 1997; Konner et al., 2007; Lin et al., 2010).

ObRb belongs to the class I cytokine receptor family, which is known to act through Janus kinases (JAKs) and signal transducers and activators of transcription (STATs). ObRb contains intracellular motifs required for the activation of the JAK/STAT signal transduction pathway (Myers, 2004), one of the main signalling cascades activated by leptin (Ahima and Osei, 2004; Sweeney, 2002). The JAK/STAT signal transduction cascade is activated by interferons, interleukins or other cytokines whose receptors lack intrinsic kinase activity. The ObRb does not have an intrinsic tyrosine kinase domain, and therefore binds cytoplasmic kinases, mainly Janus kinase 2 (JAK2) (Ghilardi and Skoda, 1997). Leptin stimulates JAK2 activation that subsequently autophosphorylates on multiple tyrosines (Banks et al., 2000; Morris and Rui, 2009). JAK2 also phosphorylates ObRb on three tyrosine residues: Tyr^{985}, Tyr^{1077}, and Tyr^{1138} (Banks et al., 2000). Phopho-Tyr^{985}, -Tyr^{1077}, and -Tyr^{1138} serve as binding sites for additional signaling molecules that contain the Src homology 2 (SH2) domain, and recruit these downstream molecules to the ObRb-JAK2 complex to allow JAK2 to phosphorylate these effector proteins (White et al., 1997). Apart from JAK2, the Src tyrosine kinase family members also appear to be involved in mediating leptin signaling independently of JAK2 (Jiang et al., 2008). Phospho- Tyr^{1138} recruits the SH2 domain of signal transducer and activator of transcript 3 (STAT3), which is subsequently phosphorylated by ObRb-associated JAK2, with the consequent dimerization and nuclear tranlocation (Vaisse et al., 1996). Thus, STAT3 dimers act as a transcription factor to regulate the expression of STAT3 target genes, including suppressor of cytokine signaling 3 (SOCS3) (Banks et al., 2000; Xu et al., 2007). These pathways act coordinately to

regulate energy balance and body weight, and a large body of genetic evidence demonstrates that the JAK2/STAT3 pathway is required for the anti-obesity effect of leptin (Zhou and Rui, 2013).

At the peripheral level, the ObRb receptor is expressed in skeletal muscle, liver, pancreas and adipose tissue, but in lower levels than the brain (Muoio and Lynis Dohm, 2002). Leptin has been described to participate in quite diverse physiological functions such as glucose and lipid homeostasis, reproduction, hematopoiesis, angiogenesis, immunity, blood pressure control, bone physiology, and tissue remodelling (Ahima and Flier, 2000b; Ducy et al., 2000; Fruhbeck, 1999, 2001; Mantzoros et al., 2011). In terms of lipid metabolism, it has been reported that leptin stimulates lipolysis in adipose tissue, by decreasing FAS expression and increasing PPARα and the enzymes of FFA oxidation (Wang et al., 1999). Moreover, leptin has been shown to repress acetyl-CoA carboxilase gene expression, fatty acid synthesis and lipid synthesis (Bai et al., 1996). Thus, leptin is involved in lipid homeostasis by both inhibiting lipogenesis and stimulating lipolysis through a direct peripheral action (Fruhbeck, 2002).

Regarding the peripheral actions of insulin, its primary biological effect is to maintain the glucose levels in the physiological range. The binding of insulin to the α subunit of the tyrosine kinase receptor (RTK) on the outside surface of cells induces a conformational change resulting in the autophosphorylation at several tyrosine residues present in the b subunits of the RTK, located inside the cell (Van Obberghen et al., 2001). This results in the recruitment of a lipid kinase -the phosphoinositide 3-kinase (PI3-kinase)- to the plasma membrane of cells, bringing it in the vicinity of its physiological substrate phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)P2) which it phosphorylates at the D3 position of the inositol ring to generate PtdIns(3,4,5)P3. A key downstream effector of Ptd(3,4,5)P3 is AKT, which is recruited to the plasma membrane. Activation of AKT also requires the protein kinase 3-phosphoinositide dependent protein kinase-1 (PDK1), which in combination with a kinase leads to the phosphorylation of AKT. Thus, AKT enters the cytoplasm where it leads to the phosphorilation and inactivation of glycogen synthase kinase 3 (GSK3), which has glycogen synthase as a substrate and catalyzes the final step in glycogen synthesis. Phosphorylation of glycogen synthase by GSK3 inhibits glycogen synthesis; therefore, the inactivation of GSK3 by AKT promotes glucose storage as glycogen. A key action of insulin is to stimulate glucose uptake into cells by inducing translocation of the glucose transporter (GLUT4), from intracellular storage to the plasma membrane. Both PI3-kinase and AKT are known to play a role in GLUT4 translocation (Lizcano and Alessi, 2002). Moreover, the regulation of GLUT4 to the plasma membrane is also performed by an independent pathway, where PI3-kinase is responsible for providing a second signal (Saltiel and Kahn, 2001). Insulin also stimulates lipogenesis and protein synthesis. Almost all cell types are responsive to insulin; however, some of them are more sensitive, like muscle, liver and adipose tissues. Insulin exerts its effects by binding to its specific cell surface receptor, thereby achieving the activation of a cascade of intracellular signaling pathways, which ultimately lead to changes in glucose transport, glycogen and lipid synthesis and specific gene expression (Alper, 2000).

1.1.3.2. Insulin and leptin resistance

Insulin resistance is defined as a state in which normal elevated insulin levels produce an attenuated biological response (Cefalu, 2001); classically, this refers to impaired sensitivity to insulin mediated glucose disposal (Reaven, 2004). Insulin resistance increases with increasing BMI, waist circumference and waist-hip ratio (Aronne and Segal, 2002). This state reflects increased levels of visceral adipose tissue, which is more metabolically active than subcutaneous fat, and with regard to fatty acid turnover. It is accepted that NEFAs play a critical role in the development of insulin resistance as well as other metabolic disturbances. The increased flux of free fatty acids promotes insulin resistance at a cellular level and increases hepatic VLDL production (Giorgino et al., 2005). Adipose tissue also produces many cytokines which have been associated with insulin resistance, including those with pro-inflammatory activity, such as TNFα, interleukins, and PAI-1. The insulin resistance seen in obesity is believed to involve primarly muscle and liver, with increased adipocyte-derived NEFAs and the concomitant TG accumulation in these tissues (Perseghin et al., 2003).

The discovery of leptin (Zhang et al., 1994), together with the observation that its replacement reverses morbid obesity in leptin-deficient mice (Halaas et al., 1995; Pelleymounter et al., 1995) and also in humans with congenital leptin deficiency (CLD) (Farooqi et al., 1999), has served as a basis for proposing leptin as a treatment for common obesity. However, when patients with common obesity and leptin-resistance were treated with an analogue of the human hormone leptin, they turned out unresponsive to the treatment (Chou and Perry, 2013). Only in the case of obese subjects whose leptin concentrations decreased after weight loss, leptin treatment was found to be modestly effective (Kissileff et al., 2012). Thus, with the exception of the scare cases of congenital leptin deficiency, the effects of leptin in obese people are not clear. In fact, leptin resistance is considered as the primary risk factor for the phatogenesis of overweight and obesity (Morris and Rui, 2009). Impairment in leptin transportation, leptin signaling, and/or hypothalamic neural circuitry that regulates energy homeostasis have been proposed as some of the possible mechanisms to explain leptin resistance (Coppari and Bjorbaek, 2012). Leptin stimulates the expression of anorexigenic BDNF in the VMN via a MC4R-dependent mechanism (Xu et al., 2003). Inhibition of the BDNF/tropomyosin receptor kinase B (TrkB) pathways results in leptin resistance, hyperphagia, and obesity in both mice and human, (Xu et al., 2003). Many factors such as hyperleptinemia, inflammation, endoplasmic reticulum (ER) stress, and defective autophagy have been reported to cause leptin resistance in obesity state. Chronic exposure to high levels of circulating leptin (hyperleptinemia), causes leptin resistance, presumably by over-activating negative feedback regulators (Knight et al., 2010), such as the active form of STAT3 in POMC neurons (Ernst et al., 2009). Low grade, chronic inflammation is closely associated to metabolic disorders including obesity (Gregor and Hotamisligil, 2011). High-fat diet feeding promotes inflammation in the peripheral tissues and also in the hypothalamus (De Souza et al., 2005). ER stress has been reported to have a role in the development of leptin resistance and obesity (Hosoi et al., 2008; Zhang et al., 2008). Moreover, hypothalamic ER stress was

observed in high-fat diet fed mice (Ozcan et al., 2009). Autophagy has also been reported to be involved in the regulation of energy homeostasis (Malik et al., 2011), and it has been described that the induction of defective hypothalamic autophagy under chronic high-fat diet feeding is attributed to hyperinsulinemia and perhaps even hyperleptinemia as a result of activation of hypothalamic mTOR by leptin (Morris and Rui, 2009).

1.1.3.3. Ghrelin and its role in the regulation of energy homeostasis

Ghrelin is a gut peptide composed of 28 amino acids mostly secreted by the gastric fundus mucosa and proximal small intestine that has a potent orexigenic function (Kojima et al., 1999). It was isolated and described in 1999 by Kojima *et al*. (Kojima et al., 1999) and its specific receptor is the growth hormone secretagogue receptor 1a (GHSR1a). Peproghrelin is known as the precursor of ghrelin, and is cleaved into ghrelin and C-ghrelin, which is further cleaved into obestatin. After cleavage, ghrelin is acylated by the enzyme ghrelin O-acyltransferase (GOAT), present in the gastric mucosa. This acylation gives rise to the active form of ghrelin (acylated ghrelin), which has a half-life of about 30 min (Seim et al., 2011). The acylation of ghrelin is necessary for ghrelin to bind to its receptor (Howard et al., 1996). Besides its stimulating effect on the growth hormone, ghrelin has a role in the control of energy homeostasis, acting in an opposite way to leptin. In fact, until now, ghrelin is the only known circulating orexigenic peptide, secreted preprandially and suppressed by food intake. One of the peculiarities of ghrelin is that, althought it is a peripherally-secreted peptide in the gastrointestinal tract, it has major effects on the CNS (Kojima et al., 1999). Ghrelin reaches the hypothalamus in three different ways to exert its orexigenic action: the first one is by systematically crossing the blood-brain barrier, the second one is via vagal afferents, and the last is via local hypothalamic synthesis and secretion, thereby exerting a paracrine action (Lim et al., 2011). Via the vagus nerve or directly at the central level, ghrelin activates the neurons in the ARC secreting orexigenic peptides, NPY and AgRP, and inhibits the anorexigenic neurons secreting POMC and CART. Orexigenic signals act via adenosine monophosphate-activated protein kinase (AMPK) and increase the dopaminergic transmission from the ventral tegmental area to the nucleus accumbens, enhancing the rewards signals (Kola and Korbonits, 2009).

It is known that gastric sympathetic activation increases gastric ghrelin secretion, and noradrenaline released from sympathetic nerve terminals has been postulated as the principal factor involved in this process (Spencer et al., 2015). Both ghrelin and leptin are important appetite-regulating hormones. However, unlike leptin, ghrelin enhances appetite and increases food intake (Wren et al., 2001). Nevertheless, circulating ghrelin has been reported to be decreased in obese subjects and inversely correlated with body mass index (Tschop et al., 2001). Moreover, low ghrelin concentrations have been associated with the prevalence of insulin resistance and type 2 diabetes (Poykko et al., 2003).

1.2. IMPACT OF THE ENVIRONMENTAL CONDITIONS IN EARLY LIFE ON ENERGY BALANCE CONTROL

1.2.1. Programming of obesity

During the early critical and sensitive periods in life, the organism has the capacity to respond to environmental situations that are alien to normal development through adaptations at the cellular and molecular levels (Patel and Srinivasan, 2002). These adaptations may, however, be disadvantageous later on when the pre and postnatal environments are suboptimal. Such early adaptations to a nutritional stress or stimulus permanently change the physiology and metabolism of the organism and continue to be expressed even in the absence of the stimulus that initiated them, a process termed "metabolic programming" (Lucas, 1991). The Dutch Hunger Winter study, published in 1976, represents the emblematic example of the effects of maternal malnutrition during pregnancy on the health of offspring (Ravelli et al., 1976). It showed a greater prevalence of obesity in 19-year-old men who were conceived during the last 6 months of the 2nd World War and whose mothers experienced poor nutrition in the 1st and 2nd trimester of pregnancy (Ravelli et al., 1976). This study also provided crucial support and fundamental insights for the growing field on metabolic programming of obesity and related metabolic complications, largely studied in the following decades. Barker (Barker, 1990, 1992, 1995) was the first to postulate the relationship between low birth weight resulting from maternal undernutrition and the increased risk for adverse health outcomes, such as type 2 diabetes, hypertension and cardiovascular disease, later in adulthood, in the hypothesis of the fetal origins of adult disease (often called "Barker's hypothesis"). As result of these initial observations, the widely recognized hypothesis on the developmental origins of health and disease (DOHaD), developed from the first notion of fetal origin of adult disease, highlights the importance of adequate maternal nutrition during gestation (Godfrey and Barker, 2000). Since then, epidemiological investigations carried out in various human populations have evidenced a direct relationship between nutrition at early stages of life and its health outcomes. Among these studies, the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort has been one which identifies that early life diet is linked to later obesity and other health endpoints (Ness, 2004). Another study that showed evidence of the maternal undernutrition effects is the Helsinki study, which shows a link between prenatal and postnatal factors and type 2 diabetes (Eriksson et al., 2003). This study evidenced large differences in the incidence of Type 2 diabetes associated with small body size at birth followed by an early adiposity rebound (Eriksson et al., 2003). In addition, the study carried out by Shiell *et al*. evidenced that high intakes of protein and fat during pregnancy may impair the development of fetal pancreatic beta cells and lead to insulin deficiency in the offspring (Shiell et al., 2000).

Due to the limitation of human interventions for ethical reasons and with the objective of further investigating the relationship between maternal nutrition and how it affects the development of a higher risk of obesity in offspring, the scientific community has carried out research in different animal models with different types of nutritional

interventions (Brenseke et al., 2013; Picó et al., 2012). In this regard, a number of animals studies have also related undernutrition during fetal life, due to maternal dietary restriction by calorie, protein or specific nutrient limitation, with a phenotype of increased risk to overweight/obesity later on in offspring life (Martorell et al., 2001; Picó et al., 2012). Unlike the fetal period, later outcomes of early postnatal undernutrition have generally received less attention. The studies performed have evidenced positive or negative lasting effects of undernutrition during lactation, generally depending on the specific factors and conditions tested. Severe food restriction during the suckling period, such as that due to increasing the litter size, has been related with growth retardation (Remmers et al., 2008a; Remmers et al., 2008b), whereas mild or moderate calorie or protein restrictions in dams during lactation have been associated in offspring with lower adiposity and an improvement of insulin and leptin sensitivity later in life (Palou et al., 2011b; Zambrano et al., 2006).

Mechanisms by which environmental factors at critical periods of development may have long-term phenotypic consequences related to obesity are not clearly established yet. In this regard, the results of the investigations carried out until now have shown that the in-utero environment is extremely labile to maternal influence, which could therefore play a crucial role in the development of permanent structural changes in key organs. It has been described that alterations in the brain, specifically in the neuronal organization of the hypothalamus (Garcia et al., 2010), together with alterations found in peripheral innervation of the SNS (Garcia et al., 2011), are responsible of the impact on body weight control and future adiposity levels of the offspring. In addition, recent evidence postulates that epigenetics modifications mediate the long-term effects of early life nutrition (Geraghty et al., 2015; Kitsiou-Tzeli and Tzetis, 2017; Nagarajan et al., 2016). Epigenetics, initially defined as “the causal interactions between genes and their products which bring phenotype into being” (Waddington, 1942), is now used to refer to stably maintained mitotically (and potentially meiotically) heritable patterns of gene expression occurring without changes in DNA sequence. These modifications include DNA methylation and histone modifications such as acetylation, methylation, phosphorylation, ADP ribosylation and ubiquitination leading to chromatin remodeling. These modifications are known to be responsive to the environment throughout life. Therefore, transient nutritional stimuli occurring at critical stages of life may have long-lasting influences on the expression of genes by interacting with epigenetic mechanisms and altering the chromatin conformation and transcription factor accessibility (Hanley et al., 2010). During the differentiation processes, changes in epigenetics patterns have been described for several genes involved in development, cellular growth, differentiation, apoptosis or tissue- or sex- specific expression (Yokomori et al., 1999, 2002).

1.2.2. Gestational calorie restriction

Many studies carried out in animal models have disclosed that maternal calorie restriction during pregnancy causes negative effects on offspring metabolic programming (Picó et al., 2012). In addition, scientific evidence supports that the outcomes of maternal undernutrition during gestation are different depending on the time, type and severity of exposure to this deficient nutritional state (Picó et al., 2012; Symonds et al., 2004). The gender of the progeny is also another important factor that may affect in a different manner the outcomes observed by maternal undernutrition. In this regard, studies carried out in an animal model of 50% calorie restriction during the first two weeks of gestation evidenced that male offspring developed higher body weight and food intake than controls from the age of five weeks, while in females there were no significant differences (Jones and Friedman, 1982; Jones et al., 1984). Regarding the period of time in which calorie restriction is carried out, it has been described that the exposure to energy restriction during the last trimester of pregnancy and the first months of life, such as the lactation period, can contribute significantly less in the development of an obesity state in adulthood (Franko et al., 2009; Palou et al., 2011b).

Different models of food restriction are used to investigate mechanisms whereby gestational undernutrition predisposes to developing metabolic related diseases. Among these, a model consisting of rat dams submitted to 50% food restriction from day 10 of gestation until delivery, considered as severe calorie restriction, has been shown to be associated with intrauterine-grow restriction, but to a rapid catch-up growth after birth. In this model, both male and female rats became obese at the age of nine months (Desai et al., 2005). A less severe gestational calorie restriction, particularly 30% throughout gestation or during the first two weeks of pregnancy (Vickers et al., 2000; Vickers et al., 2005), in spite of finding no significant differences in body weight compared to controls, offspring developed hyperphagia and greater fat accumulation, especially under hyper-caloric diet. Several studies have shown that even a mild caloric restriction (20%) during the first 12 days of gestation is also capable of causing alterations in metabolic programming in both genders, leading to an impaired capacity to regulate energy homeostasis in adult life (Palou et al., 2012; Palou et al., 2010a). Among the detrimental effects derived of mild calorie restriction, defects on hypothalamic structures and factors involved in the control of food intake and energy expenditure (Garcia et al., 2010), alterations in adipose tissue sympathetic innervation (Garcia et al., 2011), circulating ghrelin concentrations and sympathetic drive to the stomach (Garcia et al., 2013), together with an impairment in insulin and leptin sensitivity (Palou et al., 2012), were observed and associated to a higher risk to develop obesity. Table 1.1 shows a summary of the reported impact that different percentages of calorie restriction, together with time and period of gestational restriction of each experimental model, have had on offspring.

Parameter/Tissue	Offspring outcome (sex; age; diet)	Percentage of restriction and period of gestation	References
Body Weight	↑ (males; from d 74 to m 6; NF and HF diet)	20%, first 12 d	Palou et al., 2010, 2012
	↑ (females; 170 d; HF diet)	30%, first 2 w	Vickers et al., 2005
	↑ (males; 5 w; NF diet)	50%, first 2 w	Jones and Friedman, 1982; Jones et al., 1984
	↑ (males and females; 9 m; NF diet)	50%, from d 10 until delivery	Desai et al., 2005
	↑ (males and females; 260 d; HF diet)	50%, whole gestation	Thompson et al., 2007
Food intake	↑ (males and females; from d 21to m 6; NF and HF diet)	20%, first 12 d	Palou et al., 2010, 2012
	↑ (males; from d 22 to d 125; NF and HF diet)	30%, whole gestation	Vickers et al., 2000
	↑ (females; 170 d; NF and HF diet)	30%, first 2 w	Vickers et al., 2005
	↑ (males; 5 w; NF diet)	50%, first 2 w	Jones and Friedman, 1982; Jones et al., 1984
Preference for HF diet	↑ (males; 5 w; NF diet)	20%, first 12 d	Palou et al., 2010
Hypothalamic structure and function	altered (males and females; d 25; NF diet)	20%, first 12 d	García et al., 2010
	altered (males; d0; NF diet) altered (males; 8th week; NF diet) altered (males; 5th month; NF diet)	20%, 2 weeks before mating whole gestation	Ramírez-López et al. 2016
Adipose tissue sympathetic innervation	↓ (males; d 25, NF diet) ↓ (males; 4th month; NF and HF diet)	20%, first 12 d	García et al., 2011
Stomach sympathetic innervation	↓ (males and females; d 25, NF diet	20%, first 12 d	García et al., 2013

Table 1.1 Overview of the main findings in offspring of animal models subjected to different percentages of calorie restriction during gestation. Sex, age and diet for the outcome reported are specified in brackets. Abbreviations: d, day; w, week; m, months; NF, normal-fat diet; HF, high-fat diet. Adapted from (Picó et al., 2012).

1.2.3. Leptin as a crucial factor during postnatal development

Human milk contains a large number of distinct bioactive factors, including leptin, which has an important role in the neonate health and development. The presence of leptin in human milk and its production by the mammary gland was first described in 1997 (Casabiell et al., 1997; Houseknecht et al., 1997). Leptin in milk is derived from a combination of that locally synthesized by the mammary epithelium and that which is transferred from maternal plasma circulation (Casabiell et al., 1997), and its concentration in milk is positively correlated with maternal adiposity (Houseknecht et al., 1997; Miralles et al., 2006; Smith-Kirwin et al., 1998; Uysal et al., 2002).

Breast milk is practically the only food eaten during the first months of postnatal life and it is assumed to match the nutritional needs of this period. There is increasing epidemiological evidence showing that breastfeeding compared with infant formula feeding confers protection to newborns against several alterations later on in life and, particularly, against obesity and related medical complications (Armstrong and Reilly, 2002; Gillman et al., 2001; Harder et al., 2005; von Kries et al., 1999). Since leptin is not present in infant formulas (O'Connor et al., 2003), leptin was proposed as a one of the components of breast milk responsible for the beneficial effects of breastfeeding compared with infant formula (Palou and Pico, 2009). In this regard, its role in breast milk in the development of neonatal metabolism has been studied since its discovery.

Leptin supplied from maternal milk, or supplied as a water solution, can be absorbed by the immature stomach of neonate rat (Casabiell et al., 1997; Oliver et al., 2002; Sanchez et al., 2005), and be transferred into the bloodstream (Casabiell et al., 1997; Sanchez et al., 2005). In fact, leptin provided from maternal milk seems to be the main source of leptin in the stomach during the lactation period, particularly during the first-half period, when gastric leptin production is low (Oliver et al., 2002). This exogenous source of leptin could exert biological effects in neonates while the endogenous leptin-synthesizing mechanisms and appetite regulatory systems are immature (Yuan et al., 1999). In this sense, animal studies provide direct evidence of the regulatory role of leptin during development (Sanchez et al., 2005). More specifically, it was first described in rats that oral administration of leptin at physiological doses to neonate rats exerts biological effects by inhibiting food intake (Sanchez et al., 2005). Moreover, leptin supplementation throughout the suckling period was found to elicit a particular metabolic imprinting, programming offspring to be protected against overweight/obesity and other metabolic alterations with age or associated with high-fat diet feeding (Pico et al., 2007; Sanchez et al., 2008). This was associated with lasting effects on the expression of hypothalamic factors involved in the control of food intake, particularly POMC, ObRb and SOCS3, suggesting increased central leptin sensitivity (Pico et al., 2007; Sanchez et al., 2008). Thus, leptin during the sucking period was proposed to be an important factor affecting the developmental events involved in the control of energy balance in later life. Leptin during the suckling period was also shown to be associated with an improvement of peripheral leptin action, which was related to a better management and partitioning of excess fuel, increased insulin sensitivity, as well as in the prevention of other metabolic disorders such as hepatic lipid accumulation due

to a high-fat diet consumption (Priego et al., 2010; Sanchez et al., 2008). These evidences described that leptin may exert many regulatory effects, both at a central and peripheral level in the protection against obesity and its metabolic-related disorders in later life, and highlight that leptin supplementation during the suckling period may help in the comprehension of the long-term effects of metabolic programming (Palou and Pico, 2009), as is shown in Figure 1.3.

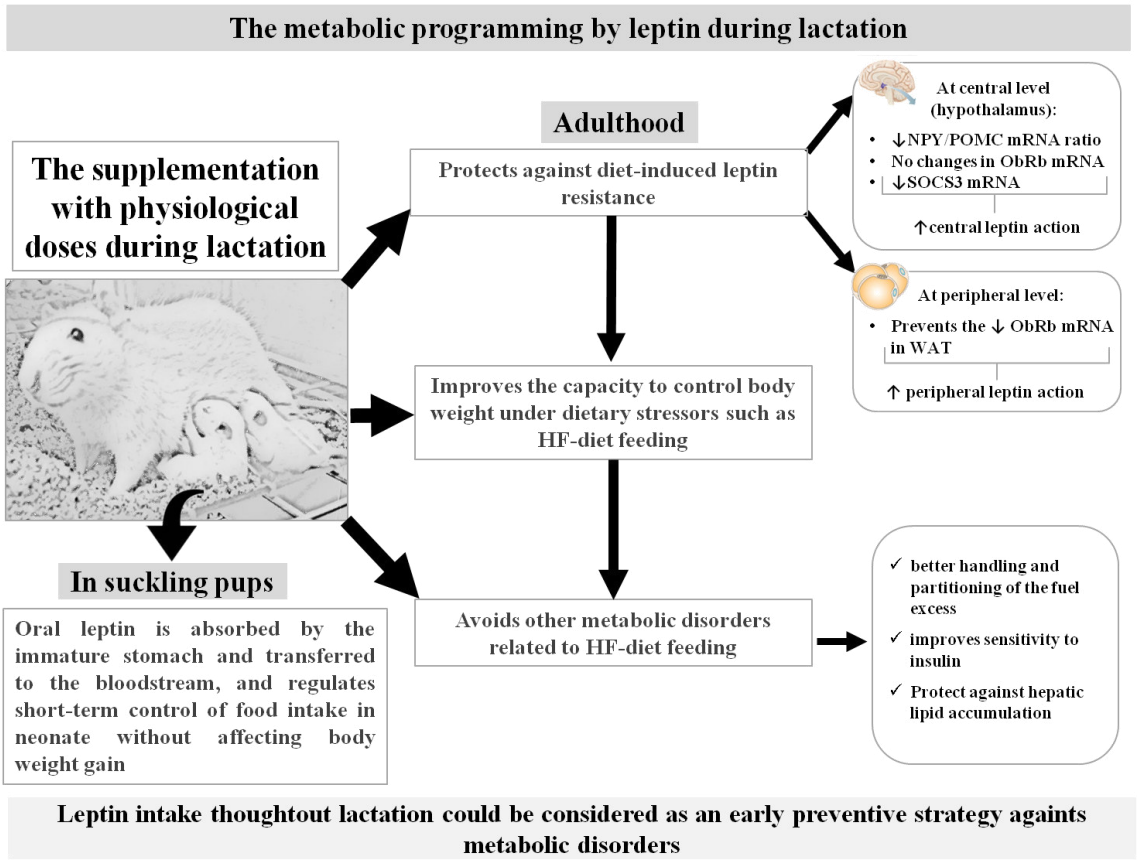

Figure 1.3. Summary of the effects in offspring of oral supplementation with physiological doses of leptin during the lactation period. In suckling pups, oral leptin is absorbed by the immature stomach and transferred to the bloodstream, and is able to regulate short-term control of food intake. In adulthood, leptin supplementation may program better adaptations to obesogenic conditions driven by an improvement in leptin action at both central and peripheral levels, which results in an improvement in the capacity to control body weight under diet stressors such as a HF-diet. Leptin supplementation also avoids other metabolic disorders related to HF-diet feeding, including alterations in hepatic metabolism and ectopic lipid accumulation. These evidences are part of the studies carried out by several authors (Pico et al., 2007; Priego et al., 2010; Sanchez et al., 2005; Sanchez et al., 2008). Abbreviations: HF: high fat; NPY: neuropeptide Y; POMC: pro-opiomelanocortin; mRNA: messenger RNA; ObRb: long form leptin receptor; SOCS3: Suppressor of cytokine signaling 3; WAT: white adipose tissue.

In humans, indirect evidences of leptin action during the suckling period have been obtained from independent epidemiological studies (Doneray et al., 2009; Miralles et al., 2006; Schuster et al., 2011). These studies show a negative correlation between milk leptin concentration and body weight gain and/or BMI increase of infants until 2 years of age (Miralles et al., 2006), the first month of life (Doneray et al., 2009) or over six months of lactation (Schuster et al., 2011). Therefore, these and other studies have

suggested that the intake of moderate amounts of leptin from maternal milk may provide moderate protection to infants from excess weight gain.

Therefore, milk leptin activity has been proposed as a nutritional factor with protective capacities during the postnatal period of life; however, mechanisms whereby leptin during lactation exert programming effects are not clearly established. Animal studies have suggested that leptin plays a neurotrophic role during the hypothalamic structures development (Bouret et al., 2004). In this regard, Ahima and collaborators described for the first time a discrete leptin surge between 7 and 10 days in neonatal mice, and that this increase is independent of fat mass or food intake (Ahima et al., 1998). This neonatal leptin surge has been linked to neuron differentiation and migration, whereas a low leptin level maintains neural progenitor cells (Udagawa et al., 2007). The site of leptin action and the precise way in which leptin can exert these effects still remains uncertain. Nevertheless, it has been postulated that a molecular mechanism involving leptin receptor, ERK and STAT3 signaling are responsible for directing formation of NPY and POMC projections (Bouret et al., 2012). Moreover, an alteration in leptin surge due to maternal undernutrition has been related to long-lasting effects on metabolism, among which are increased susceptibility to an obesogenic diet in postnatal period. Accordingly, both the magnitude and timing of leptin surge appears to be critical in the development of metabolic systems (Pico et al., 2011; Sullivan and Grove, 2010; Yura et al., 2005).

The paradigm of fetal programming, evidenced by maternal undernutrition where rodent models display the phenotype of obesity and metabolic disorders commonly observed in human populations, have begun to be used by several researchers since the last decade. Vickers et al, in the search for new strategies that could be able to revert the metabolic alterations produced in early life by maternal undernutrition during gestation, have investigated how a subcutaneous injection with pharmacological doses of leptin contributes to reverse the prenatal adaptations resulting from fetal undernutrition (Vickers et al., 2005, 2008). In addition, several studies have shown that oral treatment with physiological doses of leptin throughout lactation period is also able to revert, to some extent, the programmed effect of calorie restriction during gestation (Konieczna et al., 2013; Konieczna et al., 2015a). Thus, it has been shown that leptin treatment improves most of the altered hypothalamic structures and function (Konieczna et al., 2013), and that altered inguinal WAT innervation and function (Konieczna et al., 2015a) is also reverted by oral leptin treatment throughout lactation. These findings suggest that the exposure to neonatal leptin is capable of modulating adverse effects, leading to even reversal of alterations caused by maternal caloric restriction. This fact opens a new path in the search for nutritional components that are able to overcome the alterations in the development of metabolic programming caused by inadequate maternal nutrition.

1.3. BIOMARKERS OF ADVERSE METABOLIC PROGRAMMING

1.3.1. Biomarkers as indicators of physiological processes involved in obesity development

Prevention of chronic diseases, particularly obesity and its related alterations, is one of the major challenges in developed societies (Malik et al., 2013a). Diagnosis of the risk to these pathologies with appropriate biomarkers may help its prevention and management. However, there is a lack of reliable biomarkers of predisposition to these conditions, and hence the identification of new biomarkers could help implement preventive strategies and decrease their prevalence (Musaad and Haynes, 2007).

By definition, a biomarker (short form of biological marker) is "a biological characteristic that is objectively measured and evaluated as an indicator of normal biological or pathological processes, or a response to a therapeutic intervention" (Strimbu and Tavel, 2010). Biomarkers have been classified into two major types: biomarkers of disease and biomarkers of exposure (Mayeux, 2004). Disease biomarkers should be able to reflect the entire evolution of a disease, from the first manifestations to the latest stages. An ideal biomarker of disease should be able to predict the disease before any phenotypic signal is present in order to implement early preventive strategies (Mayeux, 2004). In turn, biomarkers of exposure should give information about the presence of an environmental substance in an organism but could also be greatly informative to know its effects on the host (Hanley et al., 2010). The utility of biomarkers of exposure in the context of metabolic programming is how they are linked to mechanistic or associative knowledge of the consequences of that exposure. The main advantage of developing robust biomarkers of exposure is that they are measurable events at a time when it is possible to produce changes in the outcome due to dietary manipulation (Hanley et al., 2010).

The biomarkers that are normally used to predict the risk of obesity and its related pathologies mainly involve body weight related measures, particularly BMI, body fat, blood pressure, lipid profile, blood glucose and insulin levels, among others. Considering the complexity of obesity and of the mechanisms contributing to obesity and its related pathologies, there is a need for multiple novel biomarkers beyond the prediction of traditional metabolic risk factors, providing information regarding increased risk to this pathology. Such biomarkers may reflect physiological changes occurring in the body before phenotypic features become evident, hence giving more time to intervene in the prevention of future disease. In this regard, there is a great interest in what could be considered health and/or prevention markers rather than disease markers, which have been addressed in the European project BIOCLAIMS (FP7-244995). The use of animal models with increased or reduced susceptibility to obesity and related alterations due to interventions during perinatal life may help obtain such biomarkers (Konieczna et al., 2015b). These biomarkers may be, in some cases, not a unique parameter, but a set of information consisting primarily of the quantitative levels of gene expression and/or protein and/or metabolites which, combined adequately, reflect the health state of a physiologically relevant process. For such purpose, the use

of global analysis techniques, known as "omics", has opened new opportunities and avenues of research in the field of biomarkers, since they can help regarding the identification, characterization and validation of such biomarkers.

1.3.2. Peripheral blood mononuclear cells (PBMCs) as a source of biomarkers of obesity and related alterations

Numerous studies carried out in the last decade have successfully used peripheral blood mononuclear cells (PBMCs) as a source of candidate transcriptomic biomarkers of health and disease (Oliver et al., 2013; Sanchez et al., 2014; Sanchez et al., 2012; Takamura et al., 2007). The use of peripheral blood cells as cellular material for the search of new biomarkers offers several advantages in comparison to the use of other tissues. These cells have been shown to respond to internal and external signals (de Mello et al., 2012). Moreover, they travel through the body and their expression levels reflect changes that occur in tissues such as liver and adipose tissue (Konieczna et al., 2014; Mohr and Liew, 2007; Oliver et al., 2013). In fact, it has been suggested that these blood cells may work as “sentinels”, and the response to physiological and pathological events occurring in different tissues are detectable as alterations in the levels of their gene transcripts (Liew et al., 2006). In addition to these biological characteristics, blood sampling supposes minimum invasiveness, it can be performed repeatedly and allows the collection of enough biological material to perform gene expression analysis.

Besides the use of PBMCs as a source of biomarkers, the study of gene expression profile in these cells is of particular interest as a source of relevant metabolic information. Alterations in PBMCs transcript levels have been related with several diseases in a way that PBMCs can reflect gene expression profiles of certain pathologies and can be used for clinical diagnosis (Liew et al., 2006). Thus, PBMCs can reflect metabolic responses of different organs to situations of increased energy demand such as fasting (Bouwens et al., 2007; Oliver et al., 2013). Also their gene expression profile has been associated with loss of body weight (Crujeiras et al., 2008; de Mello et al., 2008). Therefore, gene expression profile of PBMCs represents a powerful tool to explore the pathogenesis of disease and physiological homeostasis (de Mello et al., 2012). In fact, these cells are receiving increased attention for diagnostic purposes in humans, especially since the development of sophisticated techniques for transcriptome analysis, such as the microarray (Chaussabel et al., 2005).

Initially, studies investigating the relationship between obesity and PBMC gene expression were focused on inflammatory mediators and cytokines. This approach was logical due to the well-known association of obesity with a chronic low-grade inflammation (Gregor and Hotamisligil, 2011), which should be translated into significant changes in the expression of inflammatory mediators in the obese state when analyzed in PBMCs, as they include mainly immune cells. In this sense, it has been described that mRNA levels of cytokines (such as IL-6 and TNFα) and other inflammatory mediators (including migration inhibitor factor (MIF) and matrix metalloproteinase-9 (MMP-9)) were elevated in PBMCs of obese compared to normal

weight individuals (Ghanim et al., 2004). Thus, the expression of these genes may be used as markers of inflammatory status associated with obesity. Other authors have also found alternations in gene expression profile of inflammatory mediators in PBMCs from obese subjects (de Mello et al., 2008; O'Rourke et al., 2006). Microarray analysis, a technique of high-throughput analysis of gene expression patterns, is of special interest to identify "obesity-related biomarkers". This technique has been successfully applied in PBMCs to identify differences in the transcriptional profile between obese and lean subjects, which were not only restricted to inflammatory pathways (Ghosh et al., 2010), or in animal models of diet-induced obesity (Caimari et al., 2010). Moreover, this technique has been used to identify an altered early pattern of gene expression in PBMCs in animal models of undernutrition during gestation, which is sensitive to its reversion by leptin supplementation during the suckling period (Konieczna et al., 2015b). Therefore, the application of omics technologies combined with the used of PBMCs offers a range of opportunities in the field of biomarkers, particularly early and predictive biomarkers, which are yet to be developed.

2. HYPOTHESIS, OBJECTIVES AND EXPERIMENTAL DESIGN

2. HYPOTHESIS, OBJECTIVES AND EXPERIMENTAL DESIGN

The incidence of obesity and related pathologies is determined by both genetic and environmental factors, but conditions during the prenatal and postnatal period may lead to a different programming of the mechanisms involved in the homeostatic control of energy balance, hence affecting the susceptibility to chronic diseases. Evidence from human epidemiological studies suggests that suboptimal conditions in early life, such as maternal food restriction, may play a critical role in determining the risk of the later metabolic dysfunction. Animal studies have shown that even a mild calorie restriction during the first period of gestation exerts detrimental effects in the offspring on components of energy balance and metabolic regulation, increasing the susceptibility to develop obesity-related pathologies in adulthood, particularly when exposed to dietary stressful conditions.

These programming effects and their potential reversion require special attention, particularly when considering strategies for obesity prevention from the early stages of life. Our research group has previously described that neonatal supplementation with physiological doses of oral leptin during lactation in suckling rats has significant short- and long-term metabolic health benefits, such as a greater protection against diet-induced obesity and its metabolic-related disorders (Pico et al., 2007; Priego et al., 2010; Sanchez et al., 2005; Sanchez et al., 2008). Moreover, more recent studies have shown that leptin supplementation during lactation is able to revert the malprogrammed effects on hypothalamic and WAT structure and function observed at a juvenile age in the offspring of mild calorie-restricted dams during gestation (Konieczna et al., 2013; Konieczna et al., 2015a). However, whether the beneficial effects of leptin are long-lasting in the adult age are still unknown. The **hypothesis** of the present PhD thesis is that oral leptin supplementation during the suckling period may revert or reduce adverse malprogramming outcomes following an adverse perinatal environment and prevent the dysmetabolic phenotype in adulthood associated to this condition.

Thus, **the general objective was to study in rats whether the metabolic alterations and obesogenic risk in adulthood associated to a mild calorie restriction during gestation may be prevented by leptin supplementation throughout the suckling period. Moreover, we also aimed to use this animal model to identify potential transcript-based biomarkers in peripheral blood mononuclear cells (PBMCs) in adulthood indicative of higher risk to obesity-related metabolic alterations and sensitive to early nutritional intervention.**

The experimental work of this doctoral thesis has been performed at the Laboratory of Molecular Biology, Nutrition and Biotechnology (LBNB) - directed by Professor Andreu Palou - of the University of the Balearic Islands (UIB) and Centro de Investigación Biomédica en Red Fisiopatología de la Obesidad y Nutrición (CIBERobn). In addition, to advance in the development of this research project and to extend the PhD candidate's formation, a training period of 3 months was carried out at the laboratory of Professor Jaap Keijer, Chair of Human and Animal Physiology at Wageningen University (The Netherlands). The purpose of this stay was to become

familiar with the microarray technique and to perform whole-genome transcriptome profiling of PBMCs samples from the adult offspring of calorie-restricted dams during gestation. The PhD project was done thanks to a PhD fellowship entitled "beca para la formación de personal investigador", co-funded by the Regional Government (Conselleria d´Educació, Cultura i Universitats, CAIB) and the European Social Fund (FSE). The mentioned training stay abroad was financed with a travel grant from CIBERobn. The experiments of this PhD thesis are part of a larger research project financed by national and European bodies carried out in the LBNB, led by Prof. A Palou (AGL2012-33692; AGL2015-67019-P and BIOCLAIMS Project).

In order to achieve the principal objective of this PhD thesis, three specific objectives were set out:

1. To assess whether leptin supplementation throughout lactation in rat pups prevents the dysmetabolic phenotype in adulthood associated to mild calorie restriction during gestation and improves the metabolic adaptation to an obesogenic diet.

It has been evidenced by our group that, in rats, mild maternal calorie restriction during gestation is associated with alterations in hypothalamic structure and function in the offspring, as well as with lower sympathetic innervation in white and brown adipose tissues and stomach with lasting detrimental effects on energy balance control, particularly when animals are exposed to obesogenic conditions postnatally. Our group has already described that physiological doses of leptin (equivalent to 5 times the amount of leptin normally ingested from maternal milk) supplemented throughout lactation are able to restore hypothalamic structure, as well as the sympathetic innervation and function of WAT, which were altered in the offspring of rats exposed to mild calorie restriction during gestation. However, these effects of leptin in restoring the altered structure and function of key tissues involved in energy homeostasis were observed at a juvenile age. Here, we aimed to determine if leptin supplementation during lactation may ameliorate the altered phenotype presented by CR animals in adulthood, particularly when exposed to an obesogenic diet.

For this purpose, the offspring of ad libitum fed dams (controls), the offspring of 20% calorie restricted dams during the first 12 days of gestation (CR), and CR rats supplemented with physiological doses of leptin throughout lactation (CR-Leptin) were studied until the age of 6 months (Figure 2.1). Pups were weaned with a standard diet (SD) until 4 months of age, and then half of the animals of each group were moved to a high-fat, high-sucrose diet (Western diet, WD) until 6 months of age. Body weight and food intake were followed. At the age of 5 months, energy expenditure (indirect calorimetry), locomotive activity and blood pressure were measured. One week before sacrifice, blood samples were collected under 12h fasting conditions from the saphenous vein. Animals were killed under fed conditions at the age of 6 months by decapitation. Serum was obtained by centrifugation of blood and stored at -20°C until further analysis. Liver and WAT depots (gonadal, retroperitoneal, mesenteric and inguinal; gWAT, rWAT, mWAT and iWAT, respectively) were rapidly excised,

weighed and immediately frozen in liquid nitrogen and stored at -80°C. Circulating parameters (insulin, leptin and TG) were analyzed under fed and/or fasting conditions at the age of 6 months. Quantification of hepatic TG content and liver histological analysis was performed. The expression of genes related to energy metabolism in liver (lipogenesis, glucose uptake and metabolism, and leptin and insulin signaling) and in rWAT (fatty acid and glucose uptake, lipogenesis, lipolysis, expandability, leptin and insulin signaling, and inflammation) was analyzed. Liver and rWAT total AKT (serine/threonine protein kinase) and phosphorylated AKT on Serine 473 (pAKT) were determined by Western blot. Results from this study are included in the **Results section (4.1).**

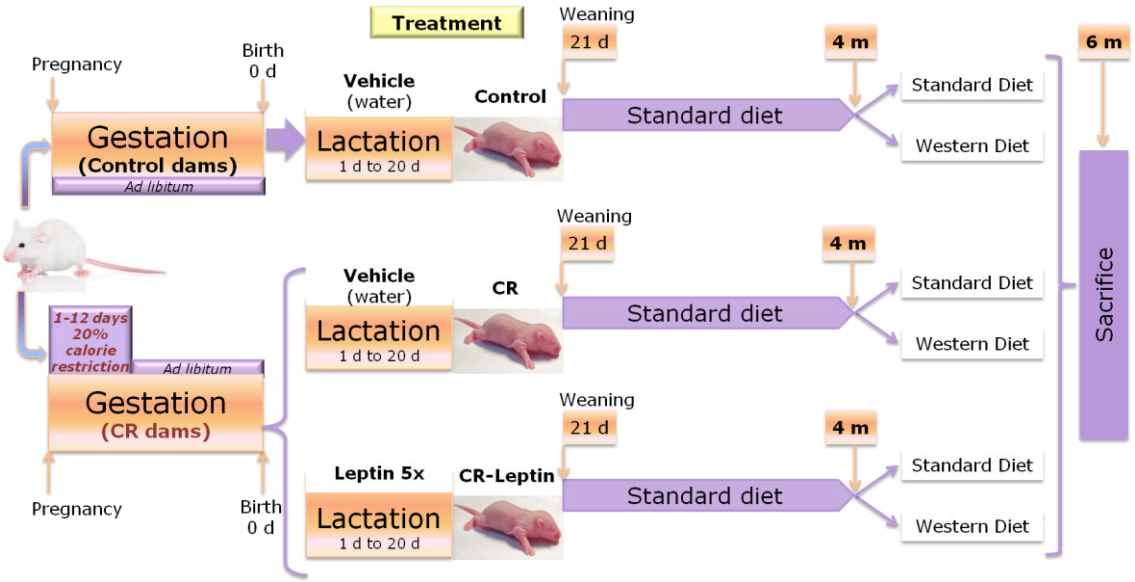

Figure 2.1. Experimental design used to achieve the first objective. The leptin dose during the suckling period is equivalent to 5 times the amount of leptin ingested normally from maternal milk (5x).

2. To assess whether leptin supplementation throughout lactation in rats delivered from dams submitted to mild calorie restriction during gestation reverses the decreased sympathetic drive to the stomach and normalizes circulating ghrelin levels.

Among the mechanisms responsible for the phenotypic alterations observed in the offspring of calorie restricted dams during gestation, our group previously reported the presence of a decreased sympathetic drive to the stomach (Garcia et al., 2013). This alteration was proposed as the responsible cause, or at least one of the contributing factors, for the presence of reduced circulating ghrelin levels in these animals, according to the involvement of sympathetic stimulation on gastric ghrelin secretion. Considering the essential role of leptin during lactation to prevent obesity and reverse developmental malprogramming effects, we aimed to investigate whether leptin supplementation during lactation is able to revert the decreased gastric sympathetic drive caused by mild gestational calorie restriction.

To meet this objective, 6 month-old control, CR and CR-Leptin animals under SD conditions belonging to the same cohort of animals described in objective 1 were used. In addition, a new cohort of animals under the same experimental conditions (controls, CR and CR-Leptin animals) was included and sacrificed at the age of 25 days (see Figure 2.2). Sympathetic innervation was determined by the analysis of tyrosine hydroxylase (TyrOH) levels and the density of TyrOH-immunoreactive ($TyrOH^+$) fibers in the stomach. In addition, circulating levels of leptin and ghrelin, both hormones with an important role in the regulation of energy balance, were determined. Results from this study are included in the **Results section (4.2)**.

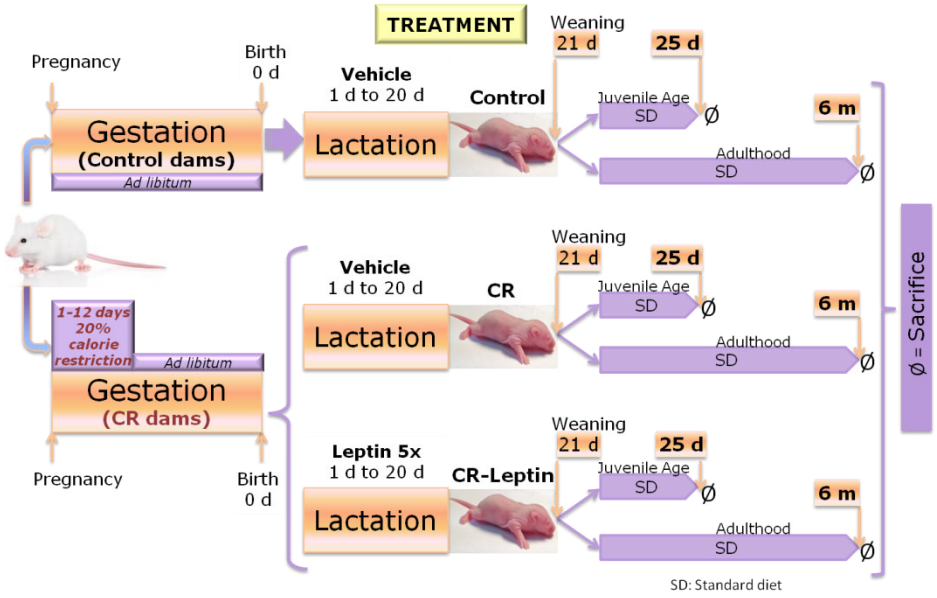

Figure 2.2. Experimental design used to achieve the second objective. The leptin dose during the suckling period is equivalent to 5 times the amount of leptin ingested normally from maternal milk (5x).

3. To identify transcriptome-based biomarkers in adulthood as potential indicators of the development of an unhealthy adult phenotype, sensitive to its reversion after leptin treatment.

Diagnosis of the risk to obesity and its related alterations with appropriate biomarkers may help its prevention and management. However, there is a lack of reliable biomarkers of predisposition to these conditions, and hence the identification of new biomarkers could help implement preventive strategies and decrease their prevalence. Within this context, peripheral blood mononuclear cells (PBMCs) are a useful source of transcriptomic biomarkers of health and disease states.

The animal models used in the present PhD thesis, whose bias was acquired due to the influence of environmental and nutritional factors at early ages (i.e. rats exposed to undernutrition during gestation and rats treated with leptin during lactation) were suggested to be useful in adulthood, but prior to the phenotypic alterations are manifested, for the identification of biomarkers predictive of increased risk to metabolic

disorders and its reversion by intervention in early life. Therefore, these animal models were used to determine potential transcript-based biomarkers in PBMCs indicative of a higher risk to develop obesity-related metabolic alterations in adulthood and sensitive to early nutritional intervention.

Thus, whole genome profiling of PBMCs from 4 month-old animals belonging to the same cohort of animals described in objective 1 (Figure 2.1) (control, CR and CR-Leptin animals) was performed using microarray technology, to identify novel potential biomarkers in adult age indicative of a higher risk to develop alterations related to the metabolic syndrome and also sensitive to its reversion by nutritional intervention during early life. Those genes whose expression levels were affected by gestational calorie restriction and reverted by leptin treatment throughout lactation were of special interest. Results from this study are included in the **Results section (4.3).**

The three objectives described above belong to the main core of my PhD project. Furthermore, at the early beginning of the PhD thesis, I collaborated in a study carried out by our group, with the objective of characterizing the effects of a mild maternal calorie restriction during gestation on the programming of BAT thermogenesis capacity in offspring at a juvenile age (25 days of age). The results of this study are included in the **Annex section (8.1)**. In summary, we reported that mild (20%) maternal calorie restriction during the first 12 days of pregnancy programs offspring for lower BAT thermogenic capacity, due to an 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.

3. GENERAL METHODS AND MATERIALS

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3.1. EXPERIMENTAL ANIMALS

The experimental design of this thesis included the use of both male and female *Wistar* rat animals from Charles River Laboratories (Barcelona, Spain). During experiments, animals were housed in the animal facilities at the University of the Balearic Islands (UIB) with a 12 h light-dark cycle. Housing temperature was 22 ºC.

To perform the animal model of mild calorie restriction during gestation, virgin female *Wistar* rats (Charles River Laboratories, Barcelona, Spain) were mated with male rats. To determine day of conception (day 0 of pregnancy), examination of vaginal smears for the presence of sperm was carried out. Pregnant rats were single caged and randomly divided into two groups: control dams were fed *ad libitum* with a standard chow diet and calorie-restricted dams (CR-dams) were subjected to a 20% calorie restriction from day 1 to day 12 of gestation. After delivery (day 1), excess pups in each litter were removed to keep 10 pups per dam, and they were randomly assigned into three groups: control, CR and CR-Leptin group. From day 1 to day 20 of lactation, and during the first 2 h of the beginning of the light cycle, 20 µl of the vehicle (water) – control and CR group – or a solution of recombinant murine leptin (PeproTech, London, England) dissolved in water – CR-Leptin group – was given orally every day to the pups using a pipette. The amount of leptin given to animals was calculated as five times the average amount of the daily leptin intake from the mother's milk (Sanchez et al., 2005). The exact daily doses for the consecutive 20 days were 1.0, 2.0, 3.0, 4.0, 5.0, 6.3, 7.5, 8.8, 10.0, 11.3, 15.6, 17.2, 18.8, 20.3, 21.9, 23.5, 25.0, 26.6, 39.4 and 43.8 ng of leptin. These doses of leptin were considered to be close to physiological levels of intake, taking into account the range of variation of milk leptin levels in dams (Sanchez et al., 2005).

Body weight and food intake of animals were recorded. Body composition was determined by using an EchoMRI-700TM (Echo Medical Systems). Adiposity index was calculated as the sum of the four fat depots measured divided by body weight and expressed as percentage, and feed efficiency was calculated as body weight gain in grams per kilocalories consumed in a specific period of time and expressed as percentage, using the formula:

$$\text{Feed efficiency (\%)} = \frac{\text{weight gained (g)}}{\text{energy consumed (kcal)}} \; 100$$

In all the experiments performed, animals were sacrificed by decapitation during the first 2 h at the beginning of the light cycle; all animals were sacrificed under *ad libitum* feeding conditions. Tissues of interest, such as the hypothalamus, liver, different white adipose tissue depots (gonadal, inguinal, mesenteric and retroperitoneal, gWAT, iWAT, mWAT and rWAT respectively) were rapidly removed, washed with 0.1 % diethyl pyrocarbonate (DEPC) saline solution (to avoid RNAses and prevent RNA degradation), frozen in liquid nitrogen and stored at -80 ºC until posterior analysis. The stomach of adult animals was removed, rinsed with saline containing 0.1 % DEPC to

remove food contents, weighed and divided longitudinally into two sections; one of them was scraped off with a glass slide and used to perform gene expression and protein analysis (obtaining approximately 0.2 g of mucosa) and the other one was used for morphometric and immunohistochemical analysis. In young animals, stomachs were cut longitudinally into two equal pieces (one for protein and the other for RNA analysis), and were immediately frozen in liquid nitrogen and stored at -80 ºC until analysis.

Reagents:

- 0.1% DEPC-saline solution: 1 ml of DEPC (Sigma) in 1 liter of saline solution.

3.1.1. Diets

Two different diets have been used depending on the experimental design: a commercial chow (Standard) diet (A04, Panlab, Barcelona, Spain) and a commercial high fat, high sucrose diet (Western Diet D12079B, Research Diet, Brogaarden, Denmark). Diets were offered under ad libitum conditions. Detailed composition of the Standard and Western diet is summarized in Table 3.1.

Description	**Panlab** **Chow diet (A04)**	**Research Diet** **Western Diet (D12079B)**
% (g/100g)	8 % of Kcal from fat	40% Kcal% fat
Carbohydrate	60.5	50
Lipid	2.9	21
Protein	15.4	20
Others (fiber, water, minerals)	21.2	9
% (Kcal/100 kcal)		
Carbohydrate	73	43
Lipid	8	40
Protein	19	17
Kcal/g	3.3	4.7
Fatty acid profile		
Saturated (%)	21.2	62.4
Monounsaturated (%)	24.8	30.7
Polyunsaturated (%)	54	13.6

Table 3.1. Composition of standard and western diet used for animal feeding.

3.1.2. Indirect gas calorimetry and locomotive activity measurements

Energy expenditure and locomotive activity were measured using the LabMaster module for indirect gas calorimetry (CaloSys) from TSE Systems. CaloSys is a fully automated high-throughput system for short- and long-term metabolic dioxide production (VCO_2) to estimate energy expenditure. Moreover, CaloSys displays a TSE InfraMot system that is designed to register the total activity of an animal under any

lighting condition by reliably sensing the heat radiating from the animal´s body and its displacement over time.

To assess energy expenditure and to quantify locomotive activity, rats were monitored for 24 h using the CaloSys. In order to reduce the potential effects of animal stress, they were individually housed and acclimated to the respiratory cages for 24 h before starting measurements. After acclimation, data on gas exchanges (VO_2, ml kg^{-1} h^{-1} and VOC_2 ml kg^{-1} h^{-1}) were measured via an open–circuit indirect calorimetry system for 24 h. To calculate energy expenditure (kcal/h), rates of oxygen consumption and carbon dioxide production were monitored for 5 min every 45 min for each animal or reference cage (our system can handle eight animal cages and one reference cage, simultaneously). Locomotive activity (counts/h) was measured continuously by the infrared beam system of the TSE InfraMot system for 24 h.

3.1.3. Blood pressure measurement

Systolic blood pressure (SBP) was determined without anesthesia using the tail-cuff method, a non-invasive blood pressure methodology. An inflatable rubber tail-cuff sphygmomanometer with a photoelectric sensor (Niprem 546, Cibertec) was placed on the animal´s tail to occlude blood flow. With this method, arterial pulsations, which are used as endpoints for indirect determination of SBP, can be detected in the rat's tail after vasodilatation. Heart rate was also registered.

Before starting the measurements, rats were placed in a rat holder and the tail-cuff sphygmomanometer and the photoelectric sensor were placed at the base of the tail for at least 30 min to acclimating animals and prevent hypertension due to stress. During the acclimation time, vasodilatation was induced by preheating the rat with a red light bulb. Signals from the tail-cuff pressure transducer and the photoelectric sensor were recorded continuously. For each rat, SBP and heart rate values were calculated as the mean of a minimum of five different measurements.

3.1.4. Blood sample collection

Blood samples were collected in heparinized tubes from the saphenous vein, without anesthesia, and during the first 2 h at the beginning of the light cycle, to obtain serum at 3 and 6 months of life. Additionally, at the end of the experimental period, and after decapitation, truncal blood samples were also obtained by centrifugation at 1000 g for 10 min at 4 ºC, saved in new sterile containers and stored at -20 ºC until analysis of circulating parameters.

3.1.5. PBMCs isolation

In order to isolate peripheral blood mononuclear cells (PBMCs), blood samples were collected from the saphenous vein at the age of 4 months under *ad libitum* feeding conditions. PBMCs were isolated from blood by density gradient separation using OptiPrep™ Density Gradient Medium. 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 of the layer contains erythrocytes which have been aggregated by the medium. The layer above erythrocytes contains mostly granulocytes, and 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 samples with OptiPrep™ (Sigma D1556) was performed according to the manufacturer´s instructions. OptiPrep™ is a medium based on iodixanol, with an adequate density and osmolality that provide a reliable and simple method for the preparation of mononuclear cells (PBMCs) from rodent and rabbit blood.

Blood samples were collected using 100 mM EDTA (pH 8.9) as an anticoagulant (the final concentration of EDTA after the addition of blood should be 4 mM, e.g. 40µl of 100 mM EDTA (pH 7.4) for 1 ml of blood were used). Then, the collected blood was diluted with solution C (solution which preserves cellular viability) to a final volume of 6 ml. Subsequently, 3 ml of density barrier were dispensed in the new tube and 6 ml of diluted blood were layered carefully over the density barrier, and centrifuged at 700 g for 20 min at room temperature with minimum acceleration and deceleration. After centrifugation, 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 PBMCs band, consisting of thrombocytes and erythrocytes. Then, the cell harvest was rinsed with two 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 a minimum acceleration and deceleration. The supernatant was removed completely and the pellet was frozen at 80 ºC until RNA extraction.

Reagents:

- OptiPrep™ Density Gradient Medium (Sigma D1556).
- 100 mM EDTA stock solution, pH 7.4: 46.53 g ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) (Sigma E-5134), dissolved in 200 ml distilled water, pH adjusted to 7.4 with NaOH and filled up to 250 ml with distilled water to obtain an EDTA solution 500mM. Then, this solution is diluted 1/5 to obtain EDTA solution 100mM.
- Solution C: 146 mM NaCl (Panreac) and 1 mM HEPES (Sigma).
- OptiPrep diluents: 2.5 volumes of Solution C diluted with 0.5 volumes of distilled water (e.g. 10 ml of Solution C mixed with 2.0 ml distilled water).
- Density barrier: 2.7 volumes of OptiPrep TM Density Gradient Medium (Invitrogen) diluted with 9.3 volumes of OptiPrep diluents (e.g. 2.7 ml OptiPrep mixed with 9.3 ml OptiPrep diluents).

3.2. MEASUREMENT OF CIRCULATING PARAMETERS

3.2.1. Glucose

Glucose levels were measured in blood using an Accu-Check Aviva system glucometer and test strips from Roche Diagnostics. To achieve this, a blood drop was obtained during blood sample collection and, after a few seconds, glucose concentration (mg/dL) appeared on the Glucometer screen.

The Accu-Chek Aviva system uses patented electrochemical methods for glucose determination. Test strips contain a capillary that sucks up 0.6 µl of blood. The glucose in the blood reacts with an enzyme electrode containing glucose dehydrogenase. Firstly, glucose is oxidized to gluconic acid and the enzyme is temporally reduced by two electrons transferred from glucose to the enzyme. The reduced enzyme reacts with an oxidized mediator, ferricyanide, transferring a single electron to each of two mediator ions. The enzyme is returned to its original state, and the two oxidized mediators are reduced to ferrocyanide. Ferricyanide and ferrocyanide are capable of rapidly transferring electrons with an electrode. Then, the Glucometer applies a potential difference between the working and counter electrodes. The counter electrode potential is defined by the ratio of ferricyanide and ferrocyanide at the electrode surface. Since the amount of ferrocyanide is small compared with the amount of ferricyanide, the concentration ratio (and hence the counter electrode potential) is effectively constant. This applied potential difference is sufficient to provide a diffusion-limited current at the working electrode, so the ferrocyanide concentration may be determined by biamperometry.

The meter measures working electrode current is linked to ferrocyanide concentration. Because the ferrocyanide concentration is coupled to glucose concentration, the current measurement permits calculation of blood glucose. Finally, data are analyzed, and the result is recorded and displayed.

Since the Accu-Chek Aviva system uses glucose dehydrogenase chemistry, glucose determination is not affected by varying degrees of sample oxygenation (capillary, venous, alternate site).

3.2.2. Insulin

Insulin levels were measured in plasma using the commercial quantitative two-site sandwich Enzyme-linked Immunoabsorbent Assay (ELISA) “Rat Insulin ELISA kit” from Mercodia.

This assay is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. 10 µl of plasma sample or calibrators were added into their respective wells and then 100 µl of enzyme conjugated 1x solution were also added into each well. The enzyme conjugated 1x solution was previously prepared according to the manufacturer´s instructions: dilution of Enzyme Conjugate 11x in Enzyme Conjugate buffer. During incubation (2 h at room temperature on a plate shaker, 700–900 rpm), insulin in the

sample reacted with peroxidase-conjugated and anti-insulin antibodies, which are bound to the micro-titer wells. After five washing steps (by aspiration and washing with Wash Buffer), every unbound molecule and unbound enzyme labeled antibody was removed. Subsequently, the bound conjugate was detected by adding 200 µl of substrate 3,3´5,5´-tetramethylbenzidine (TMB) and by incubating the microplate for 15 min at room temperature. The reaction was stopped by adding the Stop Solution to give a colorimetric endpoint that was read spectrophotometrically at 450 nm using a spectrophotometer for microplates (Tecan Sunrise Absorbance Reader).

Reagents:

- Rat Insulin ELISA (Mercodia)

3.2.3. Homeostatic model assessment for insulin resistance (HOMA-IR)

Homeostatic model assessment for insulin resistance (HOMA-IR) is a method used to quantify insulin resistance through plasmatic insulin and glucose levels, both under 14h fasting conditions. HOMA-IR was calculated using the formula of Matthews *et al*. previously described (Matthews et al., 1985).

HOMA-IR= fasting glucose (mmol/L) x fasting insulin (mU/L) / 22.5

3.2.4. Leptin

Leptin levels were measured in plasma samples using the quantitative sandwich enzyme immunoassay "Quantikine Mouse/Rat Leptin Immunoassay Kit" from R&D Systems.

The microplate provided in this kit is pre-coated with polyclonal antibody specific for mouse/rat Leptin. The assay was performed according to the manufacturer´s instructions. Firstly, 50 µl of Assay Diluent RD1W were added into each well and 50 µl of standard or sample were added to their respective wells. The microplate was incubated for 2 h at room temperature. During the incubation process, rat leptin present in the sample bound the immobilized antibody. After removing any unbound substances by five washing steps (by aspiration and washing with Wash Buffer), 100 µl of an enzyme-linked polyclonal antibody specific for mouse/rat leptin was added into each well. Microplate was incubated for 2 h at room temperature. After five washing steps, to remove any unbound antibody-enzyme reagent, 100 µl of substrate solution were added into each well, and the microplate was incubated for 30 min at room temperature. During the incubation process, the enzyme reaction yielded a blue product that turned yellow when 100 µl of the Stop Solution were added. The intensity of the color was measured at 450 nm (with wavelength correction at 540 nm) and was in proportion to the amount of leptin bound in the initial step. The sample values were then read off the standard curve. To measure the intensity of the color of each well a spectrophotometer for microplates (Tecan Sunrise Absorbance Reader) was used.

Reagents:

- Quantikine® ELISA Mouse/Rat Leptin Immunoassay (R&D Systems)

3.2.5. Adiponectin

Plasma adiponectin levels were measured using the quantitative sandwich enzyme immunoassay "Rat total Adiponectin/Acrp30 Immunoassay Kit" from R&D Systems. In this assay, a monoclonal antibody specific for rat Adiponectin had been pre-coated onto a microplate. To determine adiponectin levels in plasma, the assay was performed according to the manufacturer´s instructions. Firstly, 50 µl of Assay Diluent RD1W were added into each well and 50 µl of standard or sample were added into their respective wells. The microplate was incubated for 1 h at room temperature on a horizontal orbital shaker, during which the immobilized antibody bound any rat adiponectin present in the sample. After five washing steps (by aspiration and washing with Wash Buffer), to remove any unbound substances, 100 µl of conjugated rat adiponectin (an enzyme-linked polyclonal antibody specific for rat Adiponectin) was added to the wells. After 1 h incubation at room temperature on the shaker, five washing steps were performed to remove any unbound antibody-enzyme reagent. Then, 100 µl of Substrate Solution were added to the wells and the microplate was incubated for 30 min at room temperature on the benchtop protected from light. During the incubation process, the enzyme reaction yielded a blue product that turned yellow when 100 µl of the stop solution were added. The intensity of the color measured is in proportion to the amount of rat adiponectin bound in the initial step. Optical density of each well was determined using a spectrophotometer for microplates (Tecan Sunrise Absorbance Reader) set at 450 nm (with wavelength correction at 540 nm). The sample values were then read off the standard curve.

Reagents:

- Quantikine® ELISA Rat Total Adiponectin/Acrp30 Immunoassay (R&D Systems).

3.2.6. Ghrelin

For ghrelin determination in gastric mucosa, ghrelin peptide was extracted as described by Lee *et al.* (Lee et al., 2002) with slight modifications. Thus, the samples of mucosa were homogenized in PBS in a Teflon/glass homogenizer (Anorsa, Barcelona, Spain). The homogenate was centrifuged at 7000 *g* for 2 min at 4ºC, and the supernatant was used for ghrelin quantification. The supernatant was mixed with 10 volumes of 1 m acetic acid containing 20 mM HCl. Homogenates were boiled for 20 min and centrifuged at 7000 *g* for 2 min at 4ºC and then lyophilized and resuspended in 1:3 (wt/vol) of PBS. Ghrelin concentration in the gastric homogenates and in serum was measured with a rat ghrelin enzyme immunosorbent assay (EIA) kit (Phoenix Europe GmbH, Karlsruhe, Germany) following the manufacturer instructions.

Reagents:

- PBS: 137 mm NaCl, 2.7 mm KCl, and 10 mm phosphate buffer, pH 7.4
- 20mM HCl
- Ghrelin enzyme immunosorbent assay (EIA) kit (Phoenix Europe GmbH, Karlsruhe, Germany).

3.2.7. Triacylglyicerides (TG)

Plasma triacylglyiceride levels were determined using the enzymatic colorimetric "Serum Triacylglyiceride Determination Kit" from Sigma. The procedure of this kit involves enzymatic hydrolysis by lipase of TG to glycerol and free fatty acids. The glycerol produced is then measured by coupled enzyme reactions: firstly, glycerol is 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 dehydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2).

Subsequently, peroxidase catalyzes the coupling of H_2O_2 with 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropil) m-anisidine (ESPA) to produce a quinoneimine dye that shows an absorbance at 540 nm. The increase in absorbance at 540 nm is directly proportional to the TG concentration of the sample. Free endogenous glycerol can 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 the glycerol concentration of the sample.

This assay was performed according to the manufacturer´s instructions with some modifications in order to use the kit in a microplate format. Firstly, 240 µl of Free Glycerol Reagent were added into each well. Then, 3 µl of water, Glycerol Standard or plasma sample were added into their respective wells. The microplate was incubated for 5 min at 37 ºC and afterwards the absorbance was read at 540 nm (initial absorbance). After determination of glycerol concentrations, 60 µl of the Triacylglyiceride Reagent were added into each well and the microplate was again incubated for 5 min at 37 ºC. Finally, the absorbance was read at 540 nm (final absorbance) to determine total TG concentration. Initial and final absorbance was measured using a spectrophotometer for microplates (Tecan Sunrise Absorbance Reader). To calculate the concentration of TG in the sample, the content of glycerol was subtracted from the concentration of total TG.

To perform hepatic triacylglyicerides quantification, a piece of 100-200 mg of liver was homogenized in phosphate saline buffer (PBS) (1/3 w/v) using a polytron homogenizer. Homogenates were centrifuged (500 g, 10 min) and the supernatant was used for TG quantification with the above-referred commercial enzymatic colorimetric kit.

Reagents:

- Serum Triacylglyiceride Determination Kit (Sigma).

3.2.8. Non-esterified fatty acid (NEFA)

Plasma NEFA levels were measured using an enzymatic colorimetric method applied on kit "NEFA-HR(2) assay" from Waki Chemicals. The principle of the method is based on the fact that the NEFAs in the sample are converted to Acyl-CoA, adenosine monophosphate (AMP) and phyrophosphoric acid (PPi) by the action of Acyl-CoA synthetase (ACS), under coexistence with coenzyme A (CoA) and adenosine 5-

triphosphate disodium salt (ATP). The resultant Acyl-CoA is oxidized by the action of Acyl-CoA oxidase (ACOD). In the presence of peroxidase (POD), the hydrogen formed yields a blue purple pigment by quantitative oxidation condensation with 3-Methyl-N-Ethyl-N-(*β*-Hydroxyethyl)-Aniline (MEHA) and 4-aminoantipyrine (4-AA), which can be measured colorimetrically at 550 nm. The intensity of this pigment is proportional to the concentration of NEFA in the sample. Absorbance was measured using a spectrophotometer for microplates (Tecan Sunrise Absorbance Reader).

Reagents:

- NEFA-HR(2) assay (Wako-Chemicals).

3.3. RNA EXTRACTION

Total RNA was isolated from rWAT, liver, and stomach following either the commercial Tripure Reagent Method (Roche Applied Science) or the E.Z.N.A Total RNA kit I (Omega Bio-Tek), depending on the type of tissue and its size.

3.3.1. Tripure reagent method for RNA isolation

Approximately 200 mg of gastric mucose (adult animals) or half longitudinal section of the whole stomach (juvenile animals) was homogenized on ice with 1 ml of Tripure Reagent using a tissue homogenizer. Tripure Reagent is a mono–phasic solution of phenol and guanidine isothiocyanate that allows the maintenance of RNA integrity and denatures endogenous nucleases, while disrupting cells and dissolving cell components. To remove the fat fraction, homogenates were centrifuged at 12000 *g* for 10 min at 4 ºC, and the upper fat layer was discarded. To separate RNA, 200 µl of chloroform were added and samples were vigorously shaken for 15 s. Straightaway, a centrifugation at 12000 *g* for 15 min at 4 ºC was performed to generate three phases: a colorless aqueous upper phase which contains RNA, a white interphase with DNA and an organic pink phenol-chlorophorm lower phase which contains proteins (which was stored at -20 ºC). For RNA precipitation, the aqueous phase was transferred into a separate fresh tube and 500 µl of isopropanol were added. Samples were mixed by inversion and incubated overnight at -20 ºC. Afterwards, samples were centrifuged at 12000 *g* for 10 min at 4 ºC to precipitate RNA. Isopropanol was removed and the precipitated RNA was washed with 1 ml of 75% ethanol, vigorously shaken and centrifuged at 7600 *g* for 5 min at 4 ºC to remove ethanol completely. Finally, the precipitated RNA was dried (air-dry) for 5–10 min to evaporate any rests of ethanol. Finally, RNA pellet was resuspended in a volume of RNAase-free water and stored at -80 ºC.

Reagents:

- TriPure Reagent (Roche Applied Science)
- Chloroform (Sigma)
- Ethanol absolute (Panreac)
- RNase free water (Sigma)

3.3.2. E.Z.N.A. total RNA kit I for RNA isolation

E.Z.N.A Total RNA kit I provides a simple and rapid method to obtain purified RNA from a relatively low amount of tissue. 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 effectively washed away after a few quick wash steps.

PBMCs, 100 mg rWAT or 20 mg of liver were homogenized in 700 µl of TRK Lysis Buffer using a tissue homogenizer. Optionally, before homogenizing, 10 µl of β-

mercaptoethanol per 1 mL of TRK Lysis Buffer were added to achieve a working solution. Beta-mercaptoethanol reduces RNA degradation. To remove the fat fraction, homogenates were centrifuged at 13000 *g* for 5 min at 4 ºC. The supernatant was transferred carefully into a fresh sterile tube and 700 µl of 70% ethanol were added. Samples were vigorously shaken and the entire volume was transferred to a HiBind RNA spin column pre-inserted into a 2 mL collection tube, and then centrifuged at 10000 *g* for 2 min at room temperature. The flow-through was discarded. Then, samples were washed with 350 µl of RNA Wash Buffer I, centrifuged at 10000 *g* for 1 min and the flow-through was discarded. To avoid DNA contamination, 35 µl of DNAase I digestion reaction mix were added directly onto the surface of the membrane´s column. After incubating for 15 min at room temperature, 400 µl of RNA Wash Buffer I were added and columns were centrifuged at 10000 *g* for 1 min at room temperature. The flow-through was discarded. All columns were washed again with 500 µl of RNA Wash Buffer II and centrifuged at 10000 *g* for 1 min at room temperature. The flow-through was discarded. The final washing was performed with 350 µl of RNA Wash Buffer II, centrifuged at 10000 *g* for 1 min at room temperature and the flow-through discarded. Then, to completely dry the HiBind membrane, columns were centrifuged, with the collection tube empty, at maximum speed (20000 *g*) for 2 min. Finally, columns were transferred into a clean 1.5 mL microfuge tube and RNA was eluted with 50–100 µl of RNase free water (added directly onto the center of the membrane´s column) by centrifuging at 12000 *g* for 4 min at room temperature.

Reagents:

- Beta-mercaptoethanol
- RNA isolation kit (E.Z.N.A® Total RNA kit I) (Omega Bio-Tek)
- RNase free water (Sigma)

3.3.3. Total RNA quantification

Determination of RNA quantity and quality was performed with a NanoDrop ND-1000 UV/VA260/A280 spectrophotometer (NanoDrop Technologies) and its integrity was assessed using 1 % agarose gel electrophoresis.

NanoDrop ND-1000 spectrophotometer was used for quantification of nucleic acids using UV absorption. The concentration of nucleic acids 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 maximum absorption at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess RNA purity of an RNA precipitation. Pure RNA has an A260/A280 of 1.8–2.0; values superior to 2 indicate DNA contamination, and minor than 1.8 protein contamination. In addition, spectrophotometric measurement gives the A260/A280 ratio, which indicates the contamination degree with the organic dissolvent (the proper value is approximately 2). For spectrophotometric quantification of isolated total RNA, 2 µl of sample were used.

3.3.4. Verification of RNA integrity

The 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 a 1 % agarose gel, prepared by dissolving agarose in Tris buffer solution and adding SYBR® Safe DNA gel stain in gels. Gel was run at 85 V/cm until the blue indicator had migrated as far as 2.5 cm of the length of the gel (approximately 30 min). Bound to nucleic acids, the SYBR® Safe stain has maximum fluorescence excitation at 280 and 502 nm, and an emission maximum at 530 nm. RNA bands stained with SYBR® Safe were visualized using a UV transilluminator (ChemiGenius) connected to the program GeneSnap. After exposing the gel to UV light, the emitted fluorescence was photographed (using the GeneSnap program) and sharpness of 28S and 18S rRNA bands were evaluated.

Reagents:

- RNase free water (Sigma) or DEPC-treated water (Sigma) (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: 44.5 mM Tris base, 44.5 mM Boric acid, 1 mM EDTA)
- Loading buffer: 50 % glycerol (Sigma), 50 % water, 2.5 mg/ml Bromophenol blue (Panreac)
- SYBR Safe DNA gel stain (Invitrogen)

3.4. RT-qPCR

3.4.1. Reverse transcription of total RNA to cDNA (RT)

With the objective of determine the expression levels of selected genes evaluated in each experiment, different protocols were assessed to transcribe total RNA to cDNA, dependent on the nature of the RNA samples. RT was performed for RNA samples isolated from adipose tissue, hypothalamus, liver, stomach and from PBMCs samples.

3.4.1.1. RT of adipose tissue, liver, and stomach

Isolated total RNA (0.25 µg diluted in 5 µl of RNase free water) of the different tissue depots was firstly denaturalized at 65 ºC for 10 min in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem). 7.5 µl or RT-mix per sample was added to proceed with the RT reaction. The RT-mix consisted of: 1.25 µl of 10x buffer, 1.25 µl of 25 mM of $MgCl_2$, 2 µl of 2.5 mM dNTPs (dATP, dTTP, dCTP, dGTP), 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 RNase free water. RT conditions were as follows: 15 min at 20 ºC, 30 min at 42 ºC and a final step of 5 min at 95 ºC and keep at 4 ºC.

Reagents:

- RNase free water (Sigma)
- Buffer 10x (Promega)
- 2.5 mM $MgCl_2$ (Promega)
- RNase inhibitor 20 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 Biosystem)

3.4.1.2. RT of PBMCs samples

Due to the lower amount of total RNA isolated from PBMCs samples, an RT protocol that strengthens the sensitivity of the technique was used. For this, 0.05 µg of PBMC total RNA was used for reverse transcription by using the iScript ^{TM} cDNA Synthesis Kit. The iScript reverse transcriptase is RNase H+, which provides greater sensitivity than RNase H- enzymes in quantitative PCR. iScript is a modified MMLV-derived reverse transcriptase, optimized for reliable cDNA synthesis of a wide dynamic range of RNA. The enzyme is provided preblended with RNase inhibitor. The unique blend of oligo(dT) and random hexamers primers in the iScript reaction mix works exceptionally well with a wide variety of targets. This blend is optimized for the production of targets <1kb in length. 4 µl of 5x iScript reaction mix, 1 µl of iScript reverse transcriptase, 5 µl

of RNase free water and 5 µl of isolated total RNA 250 ng diluted in 5 µl of RNase free water of PBMC per sample were added to complete the reaction mix (a total volume of 20 µl). RT conditions to incubate the complete reaction mix were as follows: 5 min at 25 ºC, 30 min at 42 ºC, 5 min at 85 ºC and kept at 4 ºC.

Reagents:

- RT Kit (iScript ™ cDNA Synthesis Kit) (Bio-Rad Laboratories)
- RNase free water (Sigma)

3.4.2. Real-time polymerase chain reaction (qPCR)

cDNA obtained by reverse transcription was used to quantify mRNA levels of selected genes by RT-qPCR. The principle of this method is that there is an increase in the fluorescence of the SYBR® Green dye during the PCR amplification process as the SYBR® Green dye binds to double-stranded amplicons, it undergoes a conformational change and emits fluorescence at an exponential intensity.

To achieve this, 2 µl of diluted (1/5 or 1/10) cDNA template were mixed with 9 µl of a reaction mix. Per each sample, the reaction mix contained: 3.1 µl of RNase free water, 0.45 µl of each forward and reverse primers (5 µM or 10 µM) and 5 µl Power SYBER Green PCR Master Mix. The nucleotide sequences of primers and amplicon size used for performing qRT-PCR are described in Table 3.2. RT-qPCR was performed using the Applied Biosystems Step OnePlus™ Real-Time PCR System (Applied Biosystems) with the following program: 10 min at 95 ºC, followed by a total of 40 two-temperature cycles (15 s at 95 ºC and 1 min at 60 ºC). 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 obtained for each amplification curve by the instrument´s software (StepOne Software version v2.2.2) and a ΔCt value was first calculated by subtracting the Ct value of the reference gene from the Ct value of each sample. Relative gene expression numbers were calculated as a percentage of control animals, using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Different housekeeping genes were used as internal controls for gene expression normalization, depending on the tissue, age and sex of animals.

Reagents:

- RNase free water (Sigma)
- Power SYBER Green PCR Master Mix (Applied Biosystems)
- Primers forward and reverse (provided by Sigma)

Gene	Forward Primer (5´to 3´)	Reverse Primer (5´to 3´)	Amplicon Size (bp)
Cd36	AATCCTCTCCCTCTCTGGTG	TAGGCAGCATGGAACTTGAC	175
Cd68	AATGTGTCCTTCCCACAAGC	GGCAGCAAGAGAGATTGGTC	233
Cpt1a	CGAGAAGGGAGGACAGAGAC	GGACACCACATAGAGGCAGAA	201
Cs	ACTCATCCTGCCTCGTCCTT	CTCTTCCCCACCTTTAGCC	296
Fasn	CGGCGAGTCTATGCCACTAT	ACACAGGGACCGAGTAAT	222
Gck	CAACTGCGAAATCACCTTCA	AGCATTTGTGGTGTGTGGAG	163
Ghrelin	CAGAAAGCCCAGCAGAGAAA	GAAGGGAGCATTGAACCTGA	144
Gk	CCGCTCTACAATGCTGTGGT	CCCCTGTCAAACTCCAAATAAG	247
Gpat	CAGCGTGATTGCTACCTGAA	CTCTCCGTCCTGGTGAGAAG	194
Insr	CTCCTGGGATTCATGCTGTT	GTCCGGCGTTCATCAGAG	242
Isr1	GCAACCGCAAAGGAAATG	ACCACCGCTCTCAACAGG	293
Leptin	TTCACACACGCAGTCGGTAT	AGGTCTCGCAGGTTCTCCAG	186
Lpl	TATGGCACAGTGGCTGAAAG	CTGACCAGCGGAAGTAGGAG	157
Mest	CTCAGCTCTCCCCTGCTCT	GCAATCACTCGATGGAACCT	202
ObRb	AGCCAAACAAAAGCACCATT	TCCTGAGCCATCCAGTCTCT	174
Pk	CTGCGGAGAAGGTTTTCTTG	ATACAGTCAGCCCCATCCAG	172
Pnpla2	TGTGGCCTCATTCCTCCTAC	AGCCCTGTTTGCACATCTCT	271
Ppara	TGTCGAATATGTGGGGACAA	AAACGGATTGCATTGTGTGA	215
Pparg	GATCCTCCTGTTGACCCAGA	TCAAAGGAATGGGAGTGGTC	164
Ppargc1a	GATCCTCCTGTTGACCCAGA	TCAAAGGAATGGGAGTGGTC	164
Scd1	ATCCCCTCCTCCAAGGTCTA	CGGGCCCATTCATATACATC	188
Slc2a4	CAGGGCAAGGATGGTAGAGCAC	TCCCCTCCTGCCTTAGTTGGTC	149
Srebf1	AGCCATGGATTGCACATTTG	GGTACATCTTTACAGCAGTG	260
Reference Gene	**Forward Primer (5´to 3´)**	**Reverse Primer (5´to 3´)**	**Amplicon Size (bp)**
Gdi	CCGCACAAGGCAAATACATC	GACTCTCTGAACCGTCATCAA	159
18S	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC	219

Table 3.2. Nucleotide sequences of primers and amplicon size used for performing qRT-PCR. Abbreviations: *Cd 36,* cluster of differentiation 36; *Cd 68,* cluster of differentiation 68; *Cpt1a*, carnitine palmitoyltransferase 1A (Liver); *Cs*, citrate synthase; *Fasn*, fatty acid Synthase; *Scd1*, stearoyl-CoA desaturase; *Gck*, glucokinase; *Ghrelin*; *Gk*, glycerol kinase; *Gpat,* glycerol-3-phosphate acyltransferase, *Isr1* insulin substrate receptor 1; *Insr*, insulin receptor; *Leptin*; *Lpl*, lipoprotein lipase; *Mest*, mesoderm specific transcript; *ObRb*, long form leptin receptor; *Pk*, pyruvate kinase; *Pnpla2*, patatin-like phospholipase domain containing 2; *Ppara*, peroxisome proliferator-activated receptor alpha; *Pparg*, peroxisome proliferator-activated receptor gamma; *Ppargc1a*, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; *Slc2a4*, solute carrier family 2 (facilitated glucose transporter), member 4; *Srebf1*, sterol regulatory element binding transcription factor; *Gdi*, GDP dissociation inhibitor 1 and *18S*.

3.5. MICROARRAY ANALYSIS

For microarray analysis, RNA from PBMC samples obtained from control, CR and CR-Leptin animals at the age of four months were used for gene expression microarray analysis. This microarray analysis was conducted in the laboratory group of Professor Jaap Keijer, in the Department of Human and Animal Physiology at Wageningen University, in The Netherlands. Whole-genome microarray analysis was performed in RNA from PBMCs 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 start with 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 experiment were validated by RT-qPCR.

3.5.1. Assessment of RNA quality and quantity

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, South Queensferry, United Kingdom) according to manufacturer´s instruction.

Quantification of RNA samples with the use of NanoDrop was performed as previously described (section 3.3.3). Regarding integrity of RNA samples the Agilent Bioanalyzer RNA assay enables the quality analysis of RNA using 1 ul of sample. The NanoChip kit accommodates 12 samples wells, gel wells and a well for an external standard (ladder). These wells are interconnected through micro-channels. During chip preparation, the microchannels are filled with a sieving polymer and fluorescent 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 a high RIN number ($\geq$ 8) were used for microarray experiment (a total number of 32 samples).

Reagents:

- Quality assessment kit (RNA 6000 Nano LabChip Kit) (Agilent Technologies, Inc., Santa Clara, CA, USA)

3.5.2. Sample preparation

In order to prepare labeling reaction, total RNA (200 ng in a total volume of 3.5 µl) of each sample (a total number of 32 samples) was firstly reverse transcribed to complementary DNA (cDNA) using the Agilent Low Input Quick Amp Labeling kit (Agilent Technologies, Inc., Santa Clara, 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 cyanine-5 (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 0.325 µl nuclease-free water; 1.6 µl 5x transcription buffer; 0.3 µl 0.1 M 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 ºC for 2 h. Subsequently, the labeled cRNA samples were purified using Rneasy Mini Spin Columns Kit (Quiagen, Venlo, The Netherlands). The incorporation of dyes and cRNA concentration was measured using the ''microarray measurement mode'' of the NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Ins., Wilmington, DE). Only samples with the highest yield (≥0.6 µg) and the highest specific activity (≥6.0 and 8.0 pmol Cy3 or Cy5 per µg of cRNA).

Reagents:

- Reverse transcription and labeling kit (Low Input Quik Amp Labeling Kit) (Agilent Technologies, Inc., Santa Clara, CA, USA)
- Purification kit (Rneasy Mini Spin Columns Kit (Quiagen, Venlo, The Netherlands)

3.5.3. Hybridization

Hybridization was performed using the Gene Expression Hybridization Kit (Agilent Technologies, Inc., Santa Clara, CA, USA). 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 (825 ng) labeled with Cy5, linearly amplified cRNA (825 ng) labeled with Cy3, 11 µl x Blocking Agent and 2.2 µl 25x Fragmentation Buffer, in a total volume of 55 µl. Then, the samples were incubated at 60 ºC 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 4 x 44K G4131F Whole Rat Genome Agilent Microarray Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) for 17 h at 65 ºC in hybridization chambers in an oven rotating at 10 rpm (Agilent Technologies, Inc., Santa Clara, CA, USA).

Reagents:

- Hybridization Kit (Agilent Technologies, Inc., Santa Clara, CA, USA)

3.5.4. 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 at 37 ºC, 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, Inc., Santa Clara, CA, USA)

Reagents:

- Gene Expression Wash Buffer Kit (Agilent Technologies, Inc., Santa Clara, CA, USA)
- Stabilization and Drying Solution (Agilent Technologies, Inc., Santa Clara, CA, USA)
- Acetonitrile (Sigma)

3.5.5. Scanning and microarray data analysis

An Agilent Microarray Scanner (Agilent Technologies, Inc., Santa Clara, CA, USA) was used to scan the arrays. The 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. All the arrays passed the quality control based on Cartesian MA plot (where the ordinate is M = log2 (red dye)/(green dye) and the abscissa is A = log2 (√(red dye x green dye)) and signal intensity distribution (Allison et al., 2006), but one sample was discarded as an outlier. Thus, in total, a dataset from 31 arrays was used in the next step of analysis. Data were imported 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, that can remove such intensity-dependent effects in the log2 (ratio) values (Yang et al., 2002). Then, the values were log2 transformed, and the target samples (Cy5) intensities were normalized based on a reference (Cy3) design, as described previously (Pellis et al., 2003). Target signals with an average intensity lower than two-fold 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 (Keijer et al., 2010). Validity of data was assured by checking biological plausibility and by independent analysis by RT-qPCR (see below) in PBMCs. Student´s t-test was performed between groups, with the first analysis between CR and Control animals. Using the significant transcripts, we next identified those that showed a non-significant differential expression between CR-Leptin and Controls representing reversion/normalization of expression. The threshold of significance for this statistical test was set at $p < 0.05$. Moreover, fold change (FC) calculation between groups of animals (CR vs controls, CR-Leptin vs CR, and CR-Leptin vs control) was performed.

Afterwards, a statistically significant list of genes was manually analyzed taking into account their biological information, which was obtained with the use of available databases including Genecards, KEGG, NCBI, Reactome, UniProt and WikiPathways, based on key biological domains, such as molecular function and biological process.

A heat map was constructed using R after a range scaling method (zero-mean centered). Principal component analysis (PCA) was performed using the Excel add-in Multibase (Numerical Dynamics, Japan).

3.5.6. Validation of microarray analysis. Real-time quantitative RT-polymerase chain reaction (RT-qPCR) analysis

Four genes were selected to validate the microarray results: agouti related neuropeptide (*Agrp*), pro-opiomelanocortin (*Pomc*), interleukin 1 b (*Il1b*) and interleukin 10 (*Il10*). These genes were selected among those with a signal in the array above the required signal quantifiable by RT-qPCR. For RT-qPCR analysis, 50 ng of PBMC total RNA was used for reverse transcription by using iScriptTM cDNA synthesis kit (Bio-Rad Laboratories, S.A., Madrid, Spain), according to the manufacturer´s protocol.

Real-time PCR was performed using the Applied Biosystem StepOnePlus^{TM} Real-Time PCR System (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 (5µM each). Primer sequences and products for the different genes are described in Table 1. All primers were purchased from Sigma Genosys (Sigma Aldrich Química, 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 15 s and 58, 60 or 62 ºC for (as appropriate for each primer pair) 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 (Livak and Schmittgen, 2001) with Ribosomal Protein L24 (*Rpl24*), Ribosomal Protein L41(*Rpl41*) and Ribosomal Protein L36a Like (*Rpl36al*) as reference genes. These reference genes were selected based on high expression and lowest coefficient of variation over all samples by microarray analysis.

3.6. MORPHOMETRIC ANALYSIS

Pieces of fresh tissues (from rWAT, liver and stomach) were collected during animal sacrifice for histological analysis. To avoid cell damage, and to preserve the integrity, tissue samples were immediately fixed by immersion in 4 % paraformaldehyde prepared in 0.1 M phosphate buffer (pH 7.4) overnight at 4 ºC. Following, they were washed in phosphate buffer and dehydrated using a graded series of ethanol (50 % ethanol for 30 min, 75 % ethanol for 30 min, 96 % ethanol for 45 min, 96 % ethanol for 45 min at room temperature and then overnight at 4 ºC; finally, three times for 60 min with absolute ethanol). Then, tissue samples were cleared in xylene (twice for 45 min) and finally embedded in paraffin into plastic bottles. Different tissues embedded in paraffin can be stored in the same bottle until sectioning. To obtain tissue sections, paraffin was firstly melted in the oven at 60 ºC overnight, to pick up the specific tissue from the bottle. Then tissues in paraffin were fixed in blocks and cutted (5 µm thick) using a microtome, and mounted on Super-Frost/Plus slides. Sections were dehydrated with series of ethanol, cleared in xylene and finally mounted with Eukit and strained with hematoxylin-eosin. Stained sections were analysed with use of optical microscope equipped with digital camera. Images from light microscopy were digitized and the area of at least 200 cells of each section was determined using Axio Vision software (Carl Zeiss Imaging Solutions, Barcelona, Spain).

Reagents:

- 4% paraformaldehyde (Sigma)
- 0.1 M phosphate buffer (pH=7.4): 0.2 M phosphate buffer (3.25 g $NaH_2PO_4.2H_2O$ (Panreac); 11.24 g Na_2HPO_4. (Panreac) dissolved in 1 liter of distilled water, pH adjusted to 7.4) diluted in distilled water 1:1.
- 50% ethanol: 92 ml of distilled water for each 100 ml of 96% ethanol absolute (Panreac)
- 75% ethanol: 28 ml of distilled water for each 100 ml of 96% ethanol (Panreac)
- 96% ethanol (Panreac)
- Ethanol absolute (Panreac)
- Xylene (Panreac)
- Paraffin wax (Sigma)
- Hematoxylin (Panreac)
- Eosin (Panreac)
- Eukit (Panreac)

3.7. IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemical analysis of TyrOH was performed in stomach samples (n=6-8 animals per group at 25 days, and n=5-8 animals per group at 6 months). Five-micrometer-thick sections of tissue were cut with a microtome and mounted in slides. The immunohistochemical demonstration of TyrOH was performed with the avidin-biotin peroxidase (ABC) method (Hsu et al., 1981). Briefly, serial sections were incubated with 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) dilution 1:200 in PBS for 24 h at 4ºC; biotinylated goat anti-rabbit 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-diaminobenzidine 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 Eukit (Panreac) and cover-slipped. Brown adipose tissue (BAT) sections were used as a positive control for TyrOH immunoreactivity. Immunoreactive TyrOH area ($TyrOH^+$) was measured interactively in each section using AxioVision 40V 4.6.3.0. Software (Carl Zeiss, Imaging Solutions GmbH, Germany). The specific immunoreactive signal of positive TyrOH fibers (number of fibers/mm^2) was recognized as the intensive brown colour in the muscular layer area in 25 day-old animals and in the inner oblique muscular layer in 6 month-old animals. Image analysis from all groups was examined in a blind fashion.

Reagents:

- Eukit (Panreac)
- 0.3 % hydrogen peroxide in methanol (Sigma)
- Phosphate buffered saline (PBS) (pH 7.4-7.6): 137 mM NaCl (Panreac), 2.7 mM KCl (Panreac), 10 mM Na_2HPO_4 (Panreac), adjusted pH 7.4 and filled up to 1 liter with destilled water
- 3,3-diaminobenzidine (DAB) (Sigma)
- ABC reagent (Vectastain ABC kit) (Vector)
- Triton X-100 (Sigma)
- Bovine Seric Albumin (BSA) (Sigma)
- Anti-TyrOH rabbit polyclonal antibody AB1542 (Chemicon International)
- Biotinylated goat antirabbit IgG (Vector)
- Ethanol absolute (Panreac)
- Xylene (Panreac)

3.8. WESTERN BLOT ANALYSIS

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

Western blot was performed to determine AKT (serine/threonine protein kinase) and phosphorylated AKT on Serine 473 (pAKT) were determined in homogenates of liver and rWAT, Tyrosine hydroxylase (TyrOH) in homogenates of gastric content (stomach).

3.8.1. Total protein isolation, quantification, and preparation

Liver and rWAT total AKT and phosphorylated AKT on Serine 473 (pAKT) were homogenized at 4 °C in 1:5 (w:v) of 1x RIPA (radio-immunoprecipitation assay buffer) lysis buffer containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher, Rockford, IL, USA) using a Polytron homogenizer (VWR). The homogenate was centrifuged (700 g, 10 min, 4 °C) and the supernatant was used for total protein, pAKT and AKT analysis.

Stomach was homogenized at 4º C in 1:3 (w/v) of PBS containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher, Rockford, IL, USA) using a Polytron homogenizer (VWR). The homogenate was centrifuged (700 g, 10 min, 4º C) and the supernatant was used for total protein and TyrOH analysis.

For liver and rWAT tissues, protein content was measured with the Pierce BCA protein assay kit, (Pierce, Rockford, IL, USA) using 2 Albumin Standard Ampules as a standard. Briefly, 25 µl of sample, blank, or standard were loaded into their respective wells of standard ELISA microplate, and then 200 µl of Working Reagent were added into wells. Microplate was covered and incubated for 30 min at 37ºC. Then, absorbance was read at 562 nm using a spectrophotometer for microplates (Tecan Sunrise Absorbance Reader).

For stomach, protein content was measured by the Bradford method. Briefly, 5 µl of sample, blank or a different volume of standard BSA were loaded into their respective wells of standard ELISA microplate, and then 250 µl of Bradford Reagent were added. After 2 min incubation, absorbance was red at 595 nm versus water as the reference, using spectrophotometer for microplates (Tecan Sunrise Absorbance Reader).

Prior to the electrophoresis, samples were prepared by solubilizing 50 µg of total protein (for total AKT and pAKT quantification) of liver and WAT, and 150 µg of total protein (for TyrOH quantification) of stomach in Laemmli sample buffer containing 5% 2-beta-mercaptoethanol (1 µl of sample buffer per each 10 µg of total protein). Samples were heated in a boiling water bath for 3 min to denature proteins.

Reagents:

- Halt™ Protease & Phosphatase Inhibitor Cocktail 100x (Thermo Fisher Scientific)
- RIPA Lysis Buffer 1x: 50 nM Tris-HCl buffer pH=7.4, 150 nM NaCl (Panreac), 0.25% deoxycholic acid (Panreac), 1% NP40 (Sigma) and 1 mM EDTA (Merck)
- Pierce BCA protein assay kit (Thermo Fisher Scientific)
- PBS buffer (pH 7.4): 137 mM NaCl (Panreac), 2.7 mM KCl (Panreac), 10 mM Na_2HPO_4 (Panreac), adjusted pH 7.4 and filled up to 1 liter with destilled 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 (Panreac) and filled up to 1liter with distilled water
- Laemmli sample buffer: 0.5M Tris-HCl (Sigma) pH=6.8, 5% SDS (Sigma), 10% Glycerol (Sigma), 5% B-mercaptoethanol (Sigma) and 1% bromophenol blue (Panreac)
- Albumin Standard Ampule, 2 mg/ml (Thermo Scientific)

3.8.2. Protein gel electrophoresis

Total protein was fractionated by using a 4–20 % precast polyacrylamide gel (Bio-Rad) with a standard Tris-glycine running buffer system. A molecular weight marker Dual color (Bio-Rad) was used. Electrophoresis was performed at 120 V for 90 min.

Reagents:

- 4-20% precast polyacrylamide gel (Criterion TMTGXTM, Bio-Rad Laboratories)
- Tris-glycine running buffer 0.5x pH=8.3, 0.025 M Tris-Base (Sigma), 0.195M glycine (Sigma) and 0.02% SDS (Sigma)
- Dual color, Plus Protein™ Standards (Bio-Rad)

3.8.3. Immunoblotting

At the end of electrophoresis, fractioned proteins were electrotransfered from the gel onto a 0.2 µm nitrocellulose membrane using a Trans-blot Turbo Transfer System (Bio-Rad). To achieve this, seven transfer papers were soaked in Trans-blot Turbo Transfer buffer solution and placed on the bottom of the cassette electrode (anode). Then, the nitrocellulose membrane, soaked also in Trans-blot Turbo Transfer buffer solution, was placed on top of soaked stack in the cassette, and the gel was placed on the membrane. Finally, seven transfer papers were soaked in Trans-blot Turbo Transfer buffer solution and placed on top of the gel. The system was closed with the cassette electrode (catode) and inserted in the instrument. Electrotransference conditions were: 7 min at 2.5 A constant, up to 25 V.

After the transfer of proteins, the membrane was blocked by incubation with Blocking Buffer Solution in TBS 1x (1:1) during 1 hour at room temperature. Then, the membrane was washed with 0.1 % Tween-20 in TBS 1x for 60 s and incubated overnight with the primary antibody specific for pAKT and AKT in liver using a rocking shaker. After incubation, the membrane was washed four times for 5 min each

wash with 0.1 % Tween 20 in TBS 1x at room temperature on a rocking shaker. Then, the membrane was incubated with secondary antibody infrared (IR)-dyed 800 or secondary antibody infrared (IR)-dyed 680, depending on the primary antibody used previously. Incubation with the secondary antibody was performed for 30 min at room temperature, protected from light and on a rocking shaker.

For IR detection, the membrane was washed four times for 5 min each wash with 0.1 % Tween-20 in TBS 1x at room temperature and on a rocking shaker. Finally, the membrane was washed again with TBS 1x for 5 min and was scanned in the 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 V.3.0 (LI-COR Biosciences). Individual features (squares) that surrounded all the fluorescent 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 the evidence of correct loading and blotting of proteins, incubation with β-Actin or HSP90 antibody was performed as reference proteins.

Reagents:

- 0.2 µm nitrocellulose membrane (Bio-Rad)
- Trans-Blot® Turbo™ Transfer buffer solution (Bio-Rad)
- Trans-Blot® Turbo™ RTA Midi Nitrocellulose Transfer Kit (Bio-Rad)
- Odyssey Blocking Buffer Solution (LI-COR Biosciences)
- TBS 10x wash buffer pH 7.4: 500ml of 1M Tris-HCl (Sigma), pH 7.4 and 300 ml of 5M NaCl (Panreac), filled up to 1 liter with distilled water
- 0.1% Tween-20 TBS 1x wash buffer: TBS 10x buffer diluted 1:10 with distilled water, 1 g Tween-20 (Sigma)
- Antibodies are described in Table 3.3

Type of antibody	Antibody	Produced in	Commercial Source	Reference number	Dilution used	Diluted in
Primary	Polyclonal anti-UCP1	rabbit	GeneTex Inc., Irvine, CA, USA	10983	1:2000	TBS 1x with 0.1% BSA (Sigma), 0.1% sodium azide (Sigma) and 0.1% Tween-20 (Sigma)
	Polyclonal anti-TyrOH	rabbit	Santa Cruz Biotechnology Inc., Dallas, TX, USA	14007	1:2000	
	Monoclonal anti-phospho-AKT (Ser 473)	mouse	Cell Signalling, Inc., CA, USA	4051	1:2000	
	Monoclonal anti-AKT antibody	rabbit	Cell Signalling, Inc., CA, USA	9272	1:2000	
	Monoclonal anti-HSP90 antibody	rabbit	Cell Signalling, Inc., CA, USA	4877	1:2000	
	Monoclonal anti-β-Actin antibody	mouse	Cell Signalling, Inc., CA, USA	3700	1:2000	
Secondary	anti-Rabbit IRDye®800CW	goat	LI-COR® Bioscience	926-32211	1:20000	Odyssey Blocking Buffer and TBS 1x with 0.1% BSA (Sigma), 0.1% sodium azide (Sigma) and 0.1% Tween-20 (Sigma)
	anti-Mouse IRDye®680RD	goat	LI-COR® Bioscience	926-68007	1:20000	

Table 3.3. Primary and secondary antibodies used for the detection of each protein of interest in the Western blot analysis performed in liver, rWAT, stomach and BAT.

3.9. STATISTICAL ANALYSIS

Data are expressed as the mean ± standard error of the mean (s.e.m.). The statistical analysis of microarray data has been described in detail in the section 3.5.5. The methods used for statistical analysis were chosen based on the experimental question involved. For multiple comparisons, either a two-way or a repeated measures analysis of variance (ANOVA) was carried out to study the effects of perinatal treatment and the type of diet (SD or WD). One-way ANOVA was used to determine differences between groups (controls, CR, CR-Leptin). This was followed by a least significance difference (LSD) *post hoc* test. Single comparisons between groups were assessed by Student's *t* test. The variance between the groups was similar (homogeneity of variance). Threshold of significance was set at $P < 0.05$, unless indicated. Analysis was performed with SPSS for Windows (SPSS version 20, Chicago, IL, USA).

4. RESULTS

4. RESULTS

4.1. CHAPTER 1

Oral leptin supplementation throughout lactation in rats prevents later metabolic alterations caused by gestational calorie restriction

Calorie-restriction during gestation in rats has been seen to produce lasting detrimental effects in the offspring, affecting energy balance control and other related metabolic functions. Our aim was to assess whether leptin supplementation throughout lactation may prevent the dysmetabolic phenotype in adulthood associated with gestational calorie restriction. Three groups of male Wistar rats were followed: the offspring of ad libitum fed dams (controls); the offspring of 20% calorie-restricted dams during gestation (CR); and CR rats supplemented with physiological doses of leptin throughout lactation (CR-Leptin). Pups were weaned with a standard diet (SD) until 4 months of age, and then half of the animals of each group were moved to a Western diet (WD) until 6 months of age. Body weight and food intake were recorded. Energy expenditure, locomotive activity, blood parameters, liver TG, and gene expression and specific proteins in liver and white adipose tissue (WAT) were measured in adulthood. Adult CR rats, but not CR-Leptin rats, displayed greater adiposity index and feed efficiency (both under SD) than controls, along with lower locomotive activity and energy expenditure, higher HOMA-IR index and greater circulating TG and leptin levels. CR animals also exhibited increased values of the respiratory exchange ratio and more severe signs of hepatic steatosis under WD than CR-Leptin animals. Gene expression analysis revealed that CR, but not CR-Leptin, animals displayed indicators of lower capacity for WAT expansion, along with decreased lipogenesis and lipolytic capacity under SD, and impaired lipogenic response of the liver to WD feeding, in accordance with diminished insulin sensitivity and WAT leptin signaling. Oral leptin supplementation in physiological doses throughout lactation in rats prevents most of the detrimental effects on energy homeostasis and metabolic alterations in adulthood caused by inadequate fetal nutrition.

4.1.1. Background

Obesity is a multifactorial disease. Its prevalence is increasing at alarming rates and has become a worldwide public health problem (WHO, 2016). Obesity, together with malnutrition, can exist side by side in low- and middle-income countries, as they are becoming increasingly urbanized (Malik et al., 2013a). Overweight and obesity are major causes of associated morbidities, such as type 2 diabetes, hypertension, cardiovascular diseases, various types of cancers and other health problems, which increase morbidity and mortality rates (Chan and Woo, 2010). Given the high cost of obesity and comorbidities in terms of quality of life and health-care expenditure, together with the difficulty of treatment, prevention strategies are paramount.

The incidence of obesity and related pathologies is determined by both genetic and environmental factors, but conditions during the perinatal period (gestation and lactation) may lead to a different programming of the mechanisms involved in the homeostatic control of energy balance, hence affecting the susceptibility to chronic diseases (Barker, 1995; Palou et al., 2010b; Picó et al., 2012; Sullivan and Grove, 2010; Vickers et al., 2000). In this sense, evidence from epidemiological (Ravelli et al., 1976) and intervention studies in animal models with different degrees of food restriction (Palou et al., 2010a; Picó et al., 2012; Thompson et al., 2007; Vickers et al., 2000) have shown that maternal undernutrition during gestation is associated with higher susceptibility to chronic diseases in later life. In rats, mild maternal calorie restriction during gestation has been associated with alterations in hypothalamic structure and function in the offspring (Garcia et al., 2010; Ikenasio-Thorpe et al., 2007), as well as with lower sympathetic innervation in white and brown adipose tissues (Garcia et al., 2011; Palou et al., 2015) and stomach (Garcia et al., 2013), with lasting detrimental effects on energy balance control, particularly when exposed postnatally to obesogenic conditions (Palou et al., 2010a).

Focusing on the prevention of obesity, the physiological role of leptin during lactation as an essential component of breast milk for the programming of mechanisms involved in energy homeostasis is of great interest (Pico et al., 2007). In rats, oral supplementation of leptin at physiological doses during the suckling period protects towards the development of obesity and associated metabolic alterations in adulthood (Pico et al., 2007; Priego et al., 2010). Besides the cause-effect demonstration in animals, studies in humans have provided limited indirect-evidence with a negative correlation between levels of leptin in the breast milk and excess body weight gain of their infants (Doneray et al., 2009; Miralles et al., 2006; Schuster et al., 2011).

Moreover, we have recently described that leptin supplementation throughout lactation was able to restore hypothalamic structure (Konieczna et al., 2013) and sympathetic innervation and function of white adipose tissue (WAT) (Konieczna et al., 2015a), which were altered in the offspring of rats exposed to moderate calorie restriction during gestation. Leptin also normalized expression levels of most of the identified potential early biomarkers of programmed obesity risk and other metabolic alterations associated with undernutrition during pregnancy in peripheral blood mononuclear cells

(PBMCs) (Konieczna et al., 2015b). The mentioned studies showing the effects of leptin restoring the altered structure and function of key tissues involved in energy homeostasis were performed at a juvenile age of animals, after weaning. However, whether leptin-supplemented animals are effectively protected against the acquired risk to obesity-related metabolic alterations in adulthood has not been addressed.

In the present study, we aimed to assess whether leptin supplementation throughout lactation in rat pups prevents the dysmetabolic phenotype in adulthood associated to mild (20%) calorie restriction during gestation and improves the metabolic adaptation to an obesogenic diet.

4.1.2. Results

Weight-related parameters

Results on body weight and the weight of fat stores and the liver at the age of 6 months, as well as body weight gain, food intake and food efficiency of the 3 groups of animals (control, CR and CR-Leptin) from 21 days to 4 months (under SD) and from 4 to 6 months (under both SD and WD) are summarized in Table 4.1.1A. At the age of 6 months, no significant differences were found between the 3 groups of animals concerning body weight or the weight of the different WAT depots studied, although SD fed CR animals, but not CR-Leptin animals, presented a higher adiposity index (calculated as the sum of the four fat depots measured divided by body weight and expressed as percentage) than controls. No differences were found in body length (nose-to-anus length) between groups (data not shown). During the period from 21 days to 4 months (when all animals were under SD), CR and CR-Leptin animals showed lower cumulative food intake than controls, with no differences between groups concerning body weight gain and feed efficiency ($P < 0.05$, one-way ANOVA). During the 4 to 6 month period, no differences were found between groups concerning cumulative food intake, but CR animals, under SD, displayed greater feed efficiency than control and CR-Leptin animals ($P < 0.05$, one-way ANOVA). They also showed higher body weight gain than CR-Leptin animals, while control animals presented intermediate values ($P < 0.05$, one-way ANOVA). In the three groups of animals, exposure to a WD, compared with SD, resulted in higher body weight, increased weight of the different WAT depots studied and higher adiposity index at the age of 6 months ($P < 0.05$, two-way ANOVA). In turn, body weight gain, cumulative food intake and food efficiency from 4 to 6 months were also higher in animals exposed to WD compared with those exposed to SD ($P < 0.05$, two-way ANOVA). No differences were found in liver weight between groups.

Respiratory exchange ratio, energy expenditure, locomotive activity and systolic blood pressure

Figures 4.1.1 A-D show diurnal and nocturnal respiratory exchange ratio (RER) and energy expenditure, daily locomotive activity and systemic blood pressure in the three groups of animals under SD and WD at the age of 5 months. As shown in Figure

4.1.1A, CR animals exhibited a higher RER than CR-Leptin animals, while control animals presented intermediate values. WD-fed animals showed lower RER compared with values under SD ($P < 0.05$, repeated measures ANOVA), but no differences were found between groups. All groups of animals exhibited a slight but significant decrease in nocturnal RER ($P < 0.05$, repeated measures ANOVA).

Concerning energy expenditure (Figure 4.1.1B), CR animals showed lower values than controls, whereas CR-Leptin animals presented intermediate values, which were not different to those of controls ($P < 0.05$, repeated measures ANOVA). All groups of animals exhibited a nocturnal increase in energy expenditure ($P < 0.05$, repeated measures ANOVA) reflective of the characteristic diurnal feeding rhythms, with higher food intake during the dark period.

As detailed in Figure 4.1.1C, CR animals presented lower daily locomotive activity compared with control animals ($P < 0.05$, two-way ANOVA). This parameter was partially normalized in CR-Leptin animals, reaching values not different from those of controls. No differences were found concerning systolic blood pressure between groups (Figure 4.1.1D). However, CR animals exposed to WD showed a trend towards higher blood pressure compared with controls (6% increase), while CR-Leptin animals presented similar values as controls.

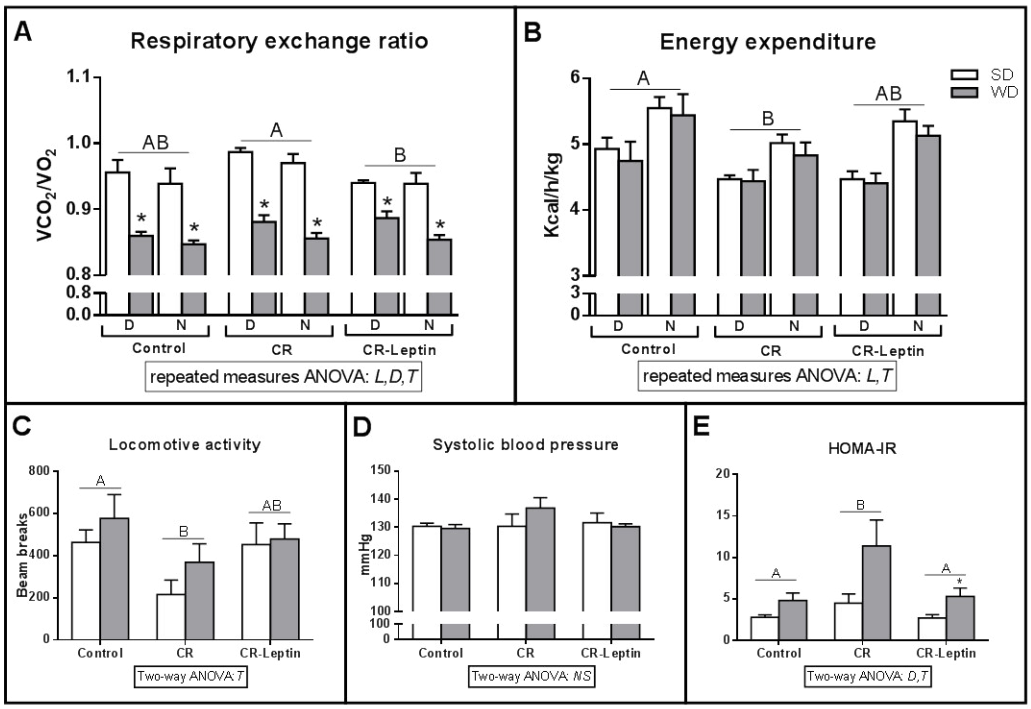


Figure 4.1.1. (A) Diurnal and nocturnal respiratory exchange ratio (RER), (B) energy expenditure, (C) daily locomotive activity, and (D) systolic blood pressure of standard diet (SD) and Western diet (WD) fed control, CR and CR-Leptin rats at the age of 5 months, and (E) HOMA-IR index of the same groups of animals at the age of 6 months. Data are mean ± S.E.M. (n=7-9). Statistical analysis between groups was performed by considering both types of diets (SD and WD) and diurnal (D) and nocturnal (N) periods. Statistics: D, effect of diet (SD and WD); T, effect of early treatment (C, CR and CR-Leptin) ($P < 0.05$, one-way or two-way ANOVA); L, effect of light/dark cycle ($P < 0.05$, repeated measures ANOVA). Data not sharing a common letter are significantly different (A ≠ B, within both SD and WD) (LSD *post hoc* analysis); *, different from their respective SD-fed group ($P < 0.05$, Student's *t*-test).

Circulating parameters

Circulating levels of glucose, insulin, leptin, adiponectin, TG and NEFA of 6-month-old animals under fed and/or fasting conditions are shown in Table 4.1.1B. Compared with controls, CR animals displayed higher fasting glucose (only under WD) ($P < 0.05$, LSD *post hoc* one-way ANOVA) and insulin levels ($P < 0.05$, two-way ANOVA), higher leptin levels (under fed conditions), higher TG levels (under fed and fasting conditions) and lower NEFA levels (under fed and fasting conditions and only under SD) ($P < 0.05$, LSD *post hoc* one-way ANOVA). These alterations were fully reversed (for glucose, insulin, and fasting TG) or partially reversed (for leptin, TG under feeding conditions and NEFA) in CR-Leptin animals ($P < 0.05$, two-way ANOVA). Consistent with these results, CR animals showed greater HOMA-IR levels than controls (60% and 137% increase under SD and WD, respectively), while CR-Leptin animals presented similar values to those of controls (Figure 4.1.1E) ($P < 0.05$, two-way ANOVA). WD-fed animals showed a higher HOMA index than those under SD ($P < 0.05$, two-way ANOVA). Adiponectin levels were not different between groups.

A. Food intake and weight related parameters							
	Control		**CR**		**CR-Leptin**		**ANOVA**
21 days to 4 months	**SD**		**SD**		**SD**		**One-way**
Body weight gain (g)	351±9		359±10		354±8		
Cumulative food intake (g)	2334±26^{a}		2258±19^{b}		2222±27^{b}		T
(Kcal)	7704±84^{a}		7450±61^{b}		7332±89^{b}		T
Feed Efficiency (%)	4.6±0.1		4.9±0.1		4.8±0.1		
4 to 6 months	**SD**	**WD**	**SD**	**WD**	**SD**	**WD**	**Two-way**
Body weight gain (g)	35.5±2.5^{ab}	78.1±5.5*	47.5±4.9^{a}	81.8±8.2*	31.7±4.6^{b}	75.3±5.6*	D
Cumulative food intake (g)	1299±31	1017±13*	1268±22	992±6*	1253±12	982±10*	D
(Kcal)	4285±102	4772±61*	4183±72	4652±28*	4136±38	4607±49*	D
Feed Efficiency (%)	0.8±0.1^{a}	1.6±0.1*	1.2±0.1^{b}	1.8±0.2*	0.8±0.1^{a}	1.6±0.1*	D
6 months	**SD**	**WD**	**SD**	**WD**	**SD**	**WD**	**Two-way**
Body weight (g)	442±21	481±14	461±20	504±12	442±18	489±11	
rWAT (g)	9.5±1.8	19.3±1.9*	13.9±1.4	20.2±1.4*	13.6±1.2	19.3±1.2*	D
gWAT (g)	9.5±1.0	16.9±1.0*	12.1±1.3	19.1±1.8*	11.6±1.0	17.2±1.0*	D
iWAT (g)	9.5±1.0	14.1±1.3*	12.1±1.2	16.2±1.8	10.9±1.2	13.8±0.9	D
mWAT (g)	5.5±0.5	8.5±0.8*	6.3±0.6	9.0±1.3	5.9±0.5	7.6±0.5*	D
Adiposity Index (%)	8.0±0.5	12.2±0.6*	9.5±0.6	13.1±1.1*	9.4±0.6	11.8±0.5*	D
Liver weight (g)	13.9±0.9	15.3±0.6	14.6±0.8	14.3±0.8	13.8±0.7	15.6±0.6*	
B. Circulating parameters at the age of 6 months							
	Control		**CR**		**CR-Leptin**		**ANOVA**
	SD	**WD**	**SD**	**WD**	**SD**	**WD**	**Two-way**
Glucose (mg/dl)							
Ad Libitum	117±4	119±6	116±5	125±10	129±5	131±7	
14-h fasting	98±4	93±2^{a}	94±4	108±3^{b,*}	92±3	100±3^{a}	DxT
Insulin (µg/L)		A		B		A	
14-h fasting	0.53±0.06	0.94±0.15*	0.80±0.21	1.76±0.46	0.51±0.07	0.89±0.16	D, T
Leptin (µg/L)		A		B		AB	
Ad Libitum	8.8±1.2	13.3±1.1*	13.3±1.7	18.3±2.5	9.2±1.2	16.3±2.2	D, T
14-h fasting	2.8±0.3	5.8±0.6	3.8±0.9	6.4±0.8	2.7±0.3	5.9±0.6	D
Adiponectin (mg/L)							
Ad Libitum	8.23±0.75	8.22±0.82	9.26±0.32	8.15±0.88	8.20±0.44	8.94±0.72	
TG (mg/ml)		A		B		AB	
Ad Libitum	1.2±0.2	2.2±0.3*	1.8±0.4	3.3±0.3*	1.4±0.2	2.5±0.3*	D, T
14-h fasting	A 0.52±0.03	A 0.72±0.11	B 0.91±0.11	B 0.86±0.05	A 0.68±0.06	A 0.66±0.04	T
NEFA (mM)							
Ad Libitum	0.59±0.05^{a}	0.79±0.11	0.41±0.06^{b}	0.83±0.12	0.51±0.05^{ab}	0.86±0.16	D
14-h fasting	0.78±0.08	0.67±0.08	0.71±0.09	0.50±0.06	0.65±0.10	0.59±0.07	

Table 4.1.1. Food intake, weight related measurements and circulating parameters. (A) Body weight gain, food intake, and feed efficiency of control, CR and CR-Leptin rats from 21 days to 4 months (under standard diet, SD) (n = 14–18) and from 4 to 6 months (under both SD and Western diet (WD)) (n = 7–9). Body weight, weight of retroperitoneal, gonadal, inguinal and mesenteric white adipose tissue (rWAT, gWAT, iWAT, and mWAT, respectively) depots, adiposity index, and liver weight at the age of 6 months are also indicated. (B) Circulating glucose, insulin, leptin, adiponectin, triacylglyicerides, and non-esterified fatty acids (NEFA) of SD and WD fed control, CR and CR-Leptin rats at the age 6 months, and under ad libitum feeding conditions and/or after 14 h fasting (n = 7–9). Data are mean ± S.E.M. Statistical analysis between groups was performed by considering both types of diets (SD and WD) ($P < 0.05$, two-way ANOVA) ($P < 0.05$, two-way ANOVA), and, separately, for each type of diet ($P < 0.05$, LSD *post hoc* one-way ANOVA) when no significant differences were found considering both types of diet

together. One-way ANOVA was also used to assess differences between groups until the age of 4 months. Symbols: T, effect of early treatment (C, CR and CR-Leptin); D, effect of diet (SD and WD) ($P < 0.05$, one-way or two-way ANOVA). For each item, data not sharing a common letter are significantly different (a ≠ b, within SD) (LSD *post hoc* analysis). *, different from their respective SD-fed group ($P < 0.05$, Student's *t*-test).

Hepatic triacylglyiceride content and histological analysis

Figure 4.1.2A shows the hepatic TG content in 6-month-old animals. CR animals had a higher TG content than CR-Leptin animals, while control animals presented an intermediate content ($P < 0.05$, two-way ANOVA). WD-fed animals exhibited greater hepatic TG content than their SD-fed counterparts ($P < 0.05$, Student's *t*-test).

Liver histological analysis (Figure 4.1.2B) revealed no signs of steatosis in SD-fed animals, regardless of the group they belonged to. However, animals exposed to WD presented increased lipid accumulation and evidence of the presence of hepatic steatosis compared with their SD-fed counterparts, particularly the control and the CR group. Of interest, WD-fed CR-Leptin animals showed lower incidence of steatosis or even no sign of it. Figure 4.1.2C includes representative images of stained liver slides belonging to animals from the different groups.

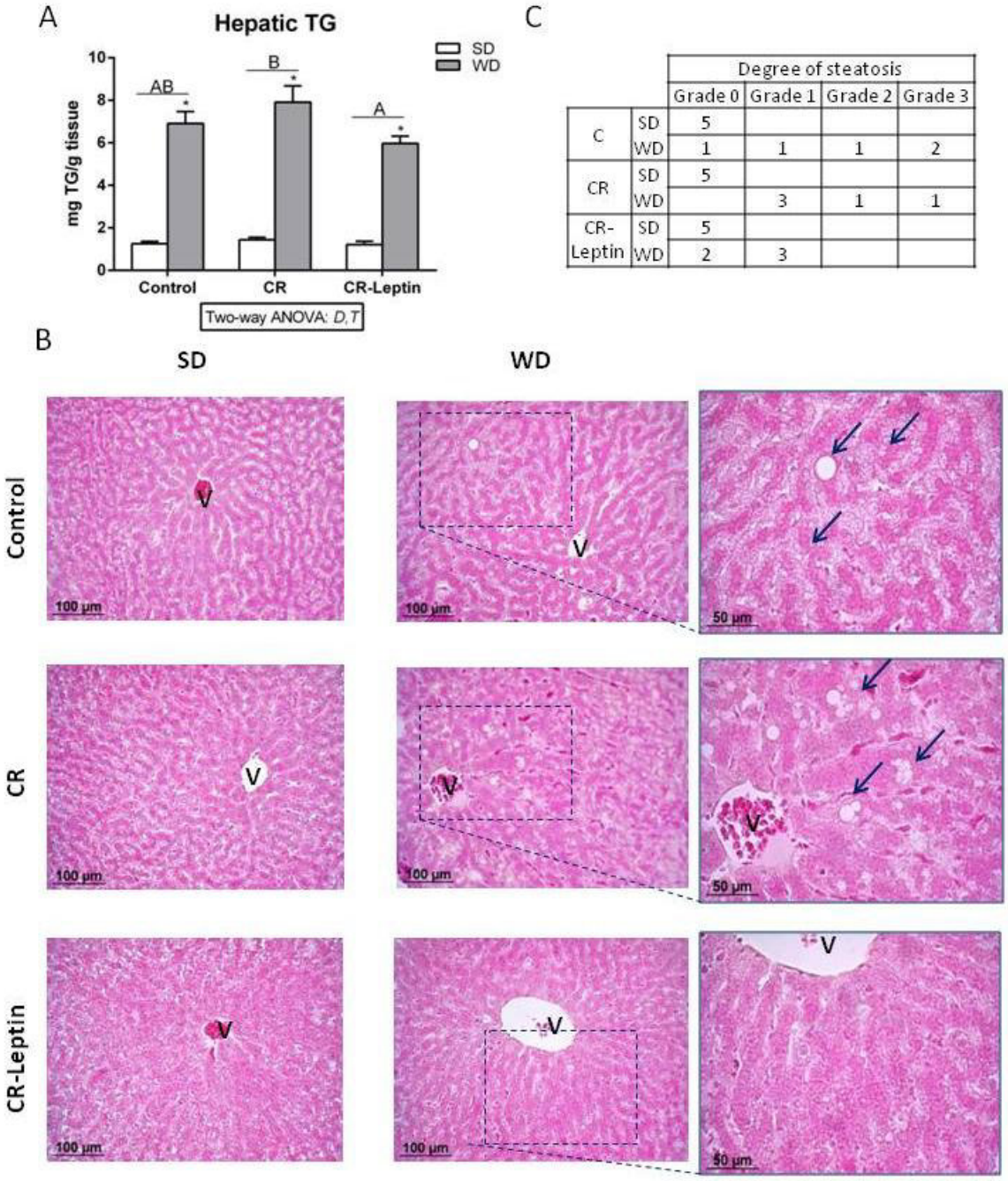


Figure 4.1.2. (A) Hepatic triacylglyiceride content, (B) representative liver slides stained with hematoxylin/eosin (x20), and (C) numerical grading of hepatic steatosis in liver biopsies from standard diet (SD) and Western diet (WD) fed control, CR and CR-Leptin rats at the age of 6 months. In A, data are mean ± S.E.M (n = 7–9). Statistical analysis between groups was performed by considering SD and WD ($P < 0.05$, two-way ANOVA). Statistics related-symbols: T, effect of early treatment (C, CR and CR-Leptin); D, effect of diet (SD and WD) ($P < 0.05$, two-way ANOVA). Data not sharing a common letter are significantly different (A ≠ B, within both SD and WD) (LSD *post hoc* analysis); *, different from their respective SD-fed group ($P < 0.05$, Student's t-test). TG, triacylglyicerides. Symbols in images: V, indicate a central vein; arrows indicate lipid droplets.

Expression of energy metabolism-related genes in liver

Hepatic mRNA expression levels of selected genes in 6-month-old animals are shown in Figure 4.1.3. No significant differences between groups were observed concerning the expression levels of the lipogenic genes *Srebf1*, *Fasn*, and *Gpat*. Regarding *Scd1*, WD-fed CR and CR-Leptin animals showed lower expression levels than controls ($P < 0.05$, LSD *post hoc* one-way ANOVA). Expression levels of most of the lipogenic genes studied (*Srebf1*, *Gpat* and *Scd1*) were affected by the type of diet in adulthood. Expression levels of Srebf1 and Scd1 were greater in WD-fed animals compared with those in SD-fed counterparts ($P < 0.05$, Student's *t*-test). Notably, in the case of *Srebf1*, the increase was greater and significant by Student's *t*-test ($P < 0.05$) in control and CR-leptin animals, but not in CR animals. In the case of *Scd1*, the increase was marked and significant by Student's *t*-test ($P < 0.05$) in control animals, but it was less marked in CR-Leptin animals and negligible in CR animals. Conversely, *Gpat* expression levels were diminished in WD-fed animals compared with levels under SD, but the decrease was more marked and significant in CR animals by Student's *t*-test ($P < 0.05$).

Regarding *Gck*, CR animals displayed higher expression levels than control and CR-Leptin animals ($P < 0.05$, two-way ANOVA). CR-Leptin animals, but not control animals, underwent an increase in the expression levels of this gene when they were exposed to WD conditions ($P < 0.05$, Student's *t*-test). Control animals showed a similar trend, but the effect was not significant (P=0.070) ($P < 0.05$, Student's *t*-test). No differences were found between groups concerning *Pk* (results not shown).

Expression levels of fatty acid oxidation-related genes (*Ppara* and *Cpt1a*) were higher in WD-exposed animals compared with their SD-fed counterparts ($P < 0.05$, two-way ANOVA), with no differences between groups (results not shown).

Concerning leptin and insulin signaling, *ObRb* mRNA levels showed an interactive effect between diet and treatment ($P < 0.05$, two-way ANOVA). WD-fed control animals displayed higher expression levels of this gene than CR and CR-Leptin animals ($P < 0.05$, two-way ANOVA), with no differences under SD. However, both control and CR-Leptin groups showed increased expression levels when exposed to WD ($P < 0.05$, Student's *t*-test), while no significant differences were found between SD- and WD-fed CR animals. Furthermore, no significant differences were observed between groups regarding *Insr* and *Irs1* mRNA expression levels.

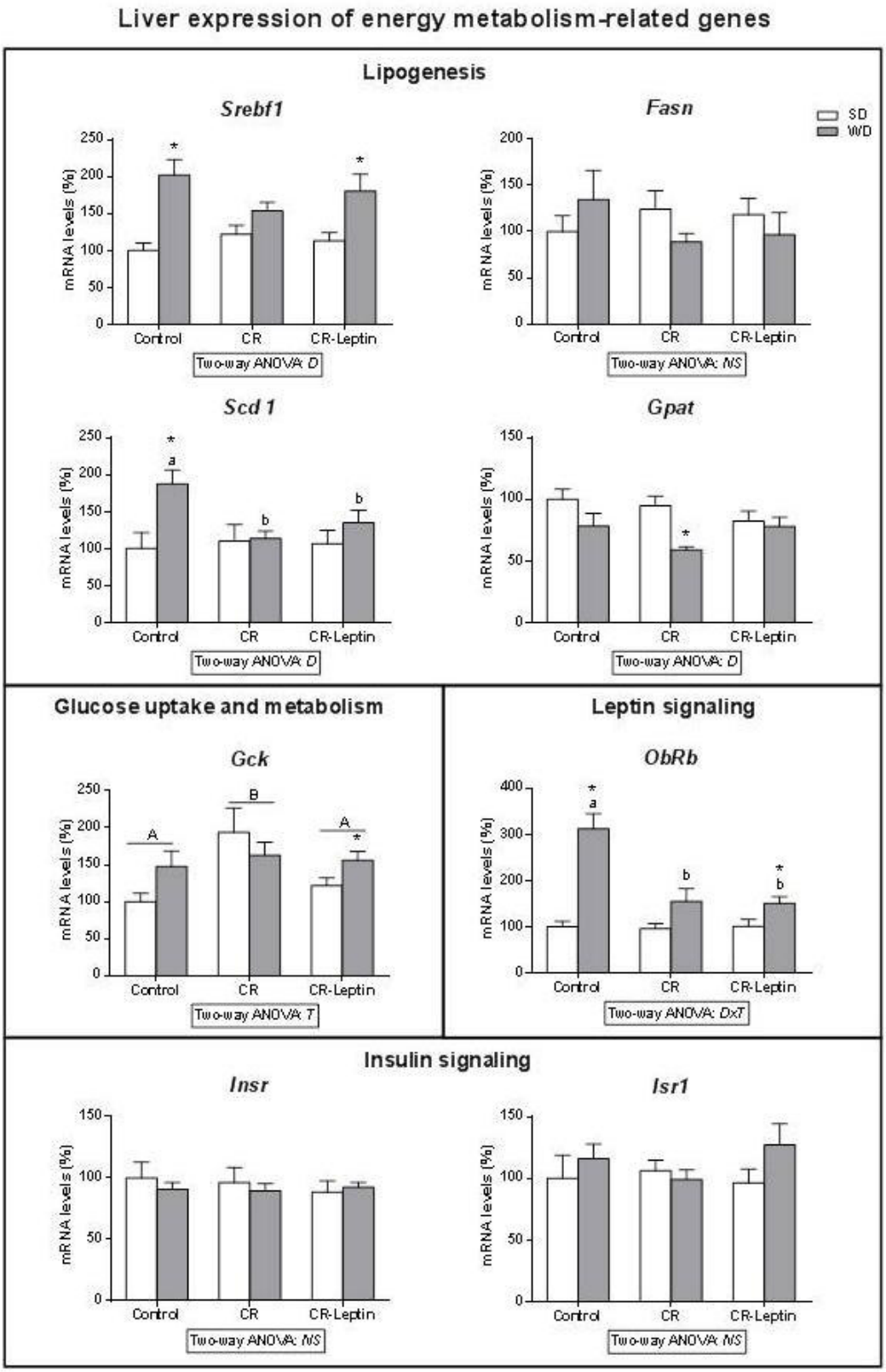


Figure 4.1.3. Expression of genes related to energy metabolism (lipogenesis, glucose uptake and metabolism, and leptin and insulin signaling) in liver from standard diet (SD) and Western diet (WD) fed control, CR and CR-Leptin rats at the age of 6 months. mRNA levels were measured by real-time PCR and expressed as a percentage of the value of control rats under SD. Data are means ± SEM (n = 7−9). Statistical analysis between groups was performed by considering both types of diets (SD and WD) ($P < 0.05$, two-way ANOVA), and, separately, for each type of diet ($P < 0.05$, one-way ANOVA) when no significant differences were found considering both types of diets together. Statistics related-symbols: D,

effect of diet (SD and WD); T, effect of early treatment (C, CR and CR-Leptin); DxT, interaction between diet and treatment ($P < 0.05$, two-way ANOVA). Data not sharing a common letter are significantly different (A ≠ B, within both SD and WD; a ≠ b, within SD or WD) (LSD *post hoc* analysis); *, different from their respective SD-fed group ($P < 0.05$, Student's t-test).

Expression of energy metabolism-related genes in rWAT

The retroperitoneal depot was chosen as a representative of WAT due to its high metabolic activity compared to other depots (Palou et al., 2010c)and because it has been related to the development of insulin resistance and type 2 diabetes (Gabriely et al., 2002). The mRNA expression levels of selected genes involved in nutrient handling and metabolism in this fat depot are shown in Figure 4.1.4. CR-Leptin animals displayed higher expression levels of *Lpl* than control and CR animals ($P < 0.05$, two-way ANOVA). Expression levels of this gene increased under WD conditions in all groups of animals, but the increase was higher and only significant in CR animals by Student's *t*-test ($P < 0.05$). No differences were found between groups regarding *Cd36* and *Slc2a4* expression levels. Under WD conditions, expression levels of both genes increased and decreased, respectively, in the different groups of animals ($P < 0.05$, two-way ANOVA).

Regarding lipogenesis-related genes, SD-fed CR animals displayed lower expression levels of *Pparg* than controls, while CR-Leptin animals showed intermediate levels which were not different to those of the other groups *(*$P < 0.05$, LSD *post hoc* one-way ANOVA). Under WD conditions, changes between groups were not apparent. *Fasn* expression levels decreased under WD conditions in all groups of animals ($P < 0.05$, two-way ANOVA), but the reduction was greater and significant by Student's *t*-test ($P < 0.05$) in control and CR-Leptin animals. Expression levels of *Gk* increased under WD conditions in all groups of animals, but the CR group showed higher expression levels of this gene compared with control and CR-Leptin animals ($P < 0.05$, two-way ANOVA). No differences were found between groups concerning *Cs* and *Ppargc1a* (data not shown). Under SD conditions, CR animals showed lower expression levels of the lipolysis-related gene *Pnpla2* compared with controls and CR-Leptin animals ($P < 0.05$, LSD *post hoc* one-way ANOVA). Expression levels of this gene increased under WD conditions in CR animals ($P < 0.05$, Student's *t*-test), and differences between groups disappeared under these dietary conditions.

Leptin expression levels were greater in WD-fed animals compared with their counterparts fed on a SD ($P < 0.05$, two-way ANOVA), with the effect being more marked and significant by Student's *t*-test in CR and CR-Leptin animals ($P < 0.05$). However, the effect in CR-Leptin was not that marked and hence WD-fed CR animals, but not CR-Leptin animals, showed higher expression levels than controls ($P < 0.05$, LSD *post hoc* one-way ANOVA). Regarding *ObRb*, CR animals presented lower expression levels than control and CR-Leptin animals ($P < 0.05$, LSD *post hoc* two-way ANOVA). Expression levels increased in all groups of animals under WD conditions, but the effect was more marked and significant by Student's *t*-test in CR and CR-Leptin animals ($P < 0.05$). No differences between groups were found concerning *Insr* and *Irs1*

expression levels, although the latter showed lower expression levels in WD-fed animals.

The expression levels of the inflammation-related gene *Cd68* were higher in CR animals compared with controls, with the CR-Leptin group showing intermediate values ($P < 0.05$, two-way ANOVA). Expression levels of this gene increased in the different groups of animals when exposed to WD ($P < 0.05$, two-way ANOVA), but the increase was more marked in control and CR-Leptin animals ($P < 0.05$, Student's *t*-test), reaching values similar to those of CR animals under NF conditions.

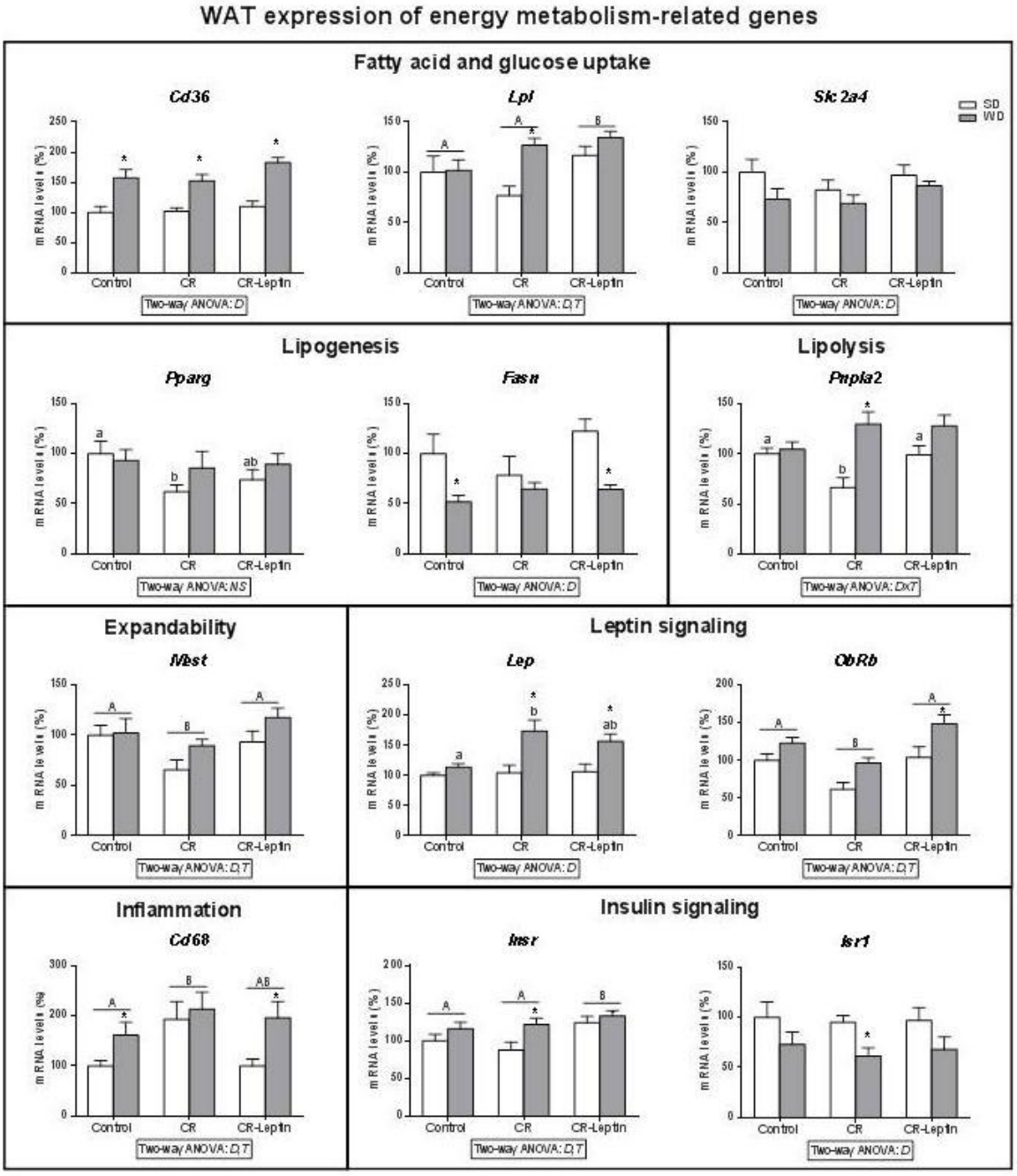


Figure 4.1.4. Expression of genes related to energy metabolism (fatty acid and glucose uptake, lipogenesis, lipolysis, expandability, leptin and insulin signaling, and inflammation) in retroperitoneal white adipose tissue from standard diet (SD) and Western diet (WD) fed control, CR and CR-Leptin rats at the age of 6 months. mRNA levels were measured by real-time PCR and expressed as a percentage of the value of control rats under SD. Data are means ± SEM (n = 7−9). Statistical analysis between groups was performed by considering both types of diets (SD and WD) ($P < 0.05$, two-way ANOVA), and, separately, for each type of diet ($P < 0.05$, one-way ANOVA) when no significant differences were found considering both types of diets together. Statistics related-symbols: D, effect of diet (SD and WD); T, effect of early treatment (C, CR and CR-Leptin); DxT, interaction between diet and treatment ($P < 0.05$, two-way ANOVA). Data not sharing a common letter are significantly different (A ≠ B, within both SD and WD; a ≠ b, within SD or WD) (LSD *post hoc* analysis); *, different from their respective SD-fed group ($P < 0.05$, Student's *t* test).

Protein levels of pAKT and AKT in liver and rWAT

Specific protein levels of pAKT and total AKT in liver are shown in Figure 4.1.5A. Animals fed a WD presented significantly lower protein levels of pAKT than those fed a SD ($P < 0.05$, two-way ANOVA), but the difference was more marked and only significant by Student's *t*-test in CR animals. On the other hand, total AKT levels showed a diet- and treatment-dependent profile; first, WD-fed animals displayed lower AKT protein levels than SD-fed ones ($P < 0.05$, two-way ANOVA). In addition, CR animals, but not CR-Leptin animals, showed significantly lower total AKT levels compared to controls ($P < 0.05$, two-way ANOVA). Concerning pAKT/AKT ratio, no significant differences were found between groups (data not shown). Specific protein levels of pAKT and total AKT in rWAT are shown in Figure 4.1.5B. Unlike what was observed in liver, animals fed a WD presented significantly higher protein levels of pAKT in rWAT than those fed a SD ($P < 0.05$, two-way ANOVA), but here again, the difference was more marked and significant by Student's t-test only in CR animals. Regarding total AKT levels, no significant differences were found between groups. Concerning pAKT/AKT ratio, WD-fed animals displayed higher levels than SD-fed animals ($P < 0.05$, two-way ANOVA), but no differences were found between groups (data not shown).

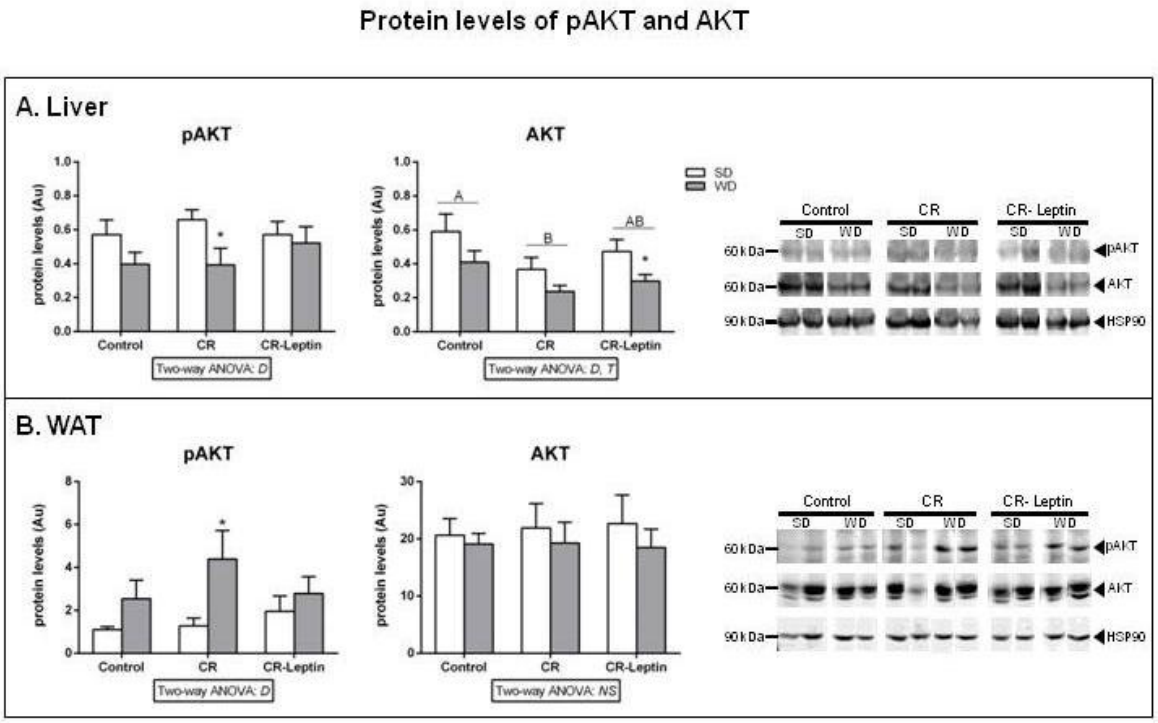


Figure 4.1.5. Protein levels of phosphoAKT (pAKT) and AKT in liver (A) and in rWAT (B) from standard diet (SD) and Western diet (WD) fed control, CR and CR-Leptin rats at the age of 6 months. Representative bands obtained are shown for pAKT, AKT and HSP90 (loading control). 50 µg of protein were loaded in each lane. Results represent mean ± SEM (n = 7-8) of ratios of specific protein levels to HSP90. Statistics related-symbols: D, effect of diet (SD and WD); T, effect of early treatment (C, CR and CR-Leptin) ($P < 0.05$, two-way ANOVA). Data not sharing a common letter are significantly different (A ≠ B, within both SD and WD) (LSD *post hoc* analysis); *, different from their respective SD-fed group ($P < 0.05$, Student's *t* test).

4.1.3. Discussion

In accordance with previous studies (Palou et al., 2012; Palou et al., 2010a; Torrens et al., 2014), present results show that mild maternal calorie restriction during the first half of gestation leads to a higher propensity of offspring to develop metabolic disturbances in adulthood. Moreover, this condition during gestation exacerbates some of the metabolic alterations associated with animal exposure to a high-fat, high-sucrose diet, the so called Western diet, as observed with other hypercaloric diets (Palou et al., 2012; Vickers et al., 2000). Interestingly, it is shown here that most of the alterations associated with undernutrition during gestation were reverted in animals supplemented with physiological doses of leptin during the suckling period. It should be noted that our experimental mild conditions of energy restriction cannot be considered rare if we extrapolate them to dieting humans; that leptin is a natural component of breast milk (Casabiell et al., 1997; Houseknecht et al., 1997), which is absent in infant formulae (O'Connor et al., 2003); and that the supplemented doses of leptin used here are physiological ones and in the range of those observed in milk (Pico et al., 2007; Sanchez et al., 2005).

The health outcomes of maternal undernutrition during gestation on the offspring, which increases obesity-related metabolic disturbances, have been widely discussed in the literature (see (Lakshmy, 2013; Picó et al., 2012). Specifically, moderate calorie restriction during gestation in rats exerts adverse effects in offspring leading to hyperphagia, increased fat accumulation, hypertension, and impaired insulin and leptin sensitivity (Palou et al., 2010a; Torrens et al., 2014; Vickers et al., 2000). Moreover, these animals display alterations in hypothalamic structure and function, showing altered expression patterns of neuropeptides regulating feeding behavior (Garcia et al., 2010; Ikenasio-Thorpe et al., 2007), as well as alterations in adipose tissue and stomach sympathetic innervations (Garcia et al., 2011; Garcia et al., 2013).

Here we have seen that the male offspring of calorie-restricted dams during gestation, despite observing no significant differences in body weight compared with controls at the age of 6 months, show higher feed efficiency and higher body fat accumulation. Measurements of energy expenditure and locomotive activity were also in favor of an imbalance in energy homeostasis in CR animals toward a positive energy balance, in accordance with previous results (Torrens et al., 2014), hence favoring fat accumulation. Interestingly, most of these alterations were normalized in the CR animals that were supplemented with oral leptin during the sucking period.

Besides differences in energy expenditure, the increased fat accumulation occurring in CR animals compared to controls and CR-Leptin animals could be related with a decreased use of fat as a metabolic substrate, as deduced from the RER values under SD conditions (near 1 during the diurnal period). Within this context, CR animals also showed diminished circulating NEFA levels, suggesting lower capacity of fat mobilization. The decreased expression levels of the lipolysis-related gene Pnpla2 and the trend towards lower expression levels of Lpl in WAT of SD-fed CR animals compared with control and CR-Leptin animals support these observations.

Fetal programming of insulin and leptin resistance due to undernutrition during the gestational period has been suggested as one of the key mechanisms responsible for energy homeostasis dysregulation occurring later in life (Palou et al., 2010a). In accordance with this, adult CR animals, but not CR-Leptin animals, displayed higher HOMA-IR index and higher plasma leptin levels than controls. Although this was observed in a more exacerbated way under WD conditions, it was also seen for SD. This is in agreement with previous results (Palou et al., 2012; Palou et al., 2010a), showing that peripheral insulin resistance in the offspring of calorie-restricted animals during gestation is programmed early and may be a direct consequence of the adverse fetal environment.

Diminished insulin sensitivity in CR animals could be associated to a dysregulation in lipogenesis in a relevant homeostatic tissue, such as the liver, due to an impaired adaptation to a hypercaloric diet. CR animals, unlike control and CR-Leptin animals, did not present a significant increase in Srebp1c mRNA levels in liver when exposed to WD conditions. SREBP1c mediates the regulating effects of insulin on the expression of lipogenic genes, such as *Gck*, *Fasn*, *Scd1*, and *Gpat* (Postic and Girard, 2008). In this regard, WD-fed CR animals did not show increased expression levels of *Gck* and *Scd1*, as observed in control animals; moreover, they exhibited a significant decrease in *Gpat* expression levels and a trend towards a decrease in *Fasn* expression levels, in agreement with the impairment in insulin sensitivity present in these animals. CR animals also displayed higher expression levels of *Gck* than controls under SD conditions, in accordance with an increased use of glucose as fuel, as suggested by their RER values. However, CR animals did not display greater expression levels of the mentioned gene under WD conditions, probably because the use of glucose may be compromised by the decreased insulin sensitivity that these animals suffer. Notably, the expression pattern of energy metabolism-related genes in liver of CR-Leptin animals was more similar to that of controls, in agreement with the normalization of circulating parameters, and the lower presence of signs of hepatic steatosis when exposed to WD conditions.

To further study insulin signaling of CR animals, total and phosphorylated AKT levels in liver were determined. That the interaction of insulin and its receptor causes insulin receptor substrate phosphorylation and that it initiates a signaling cascade which culminates in the phosphorylation of AKT is a well-known fact (Choi and Kim, 2010). Once AKT is activated, it uses different downstream pathways to modulate metabolism, resulting in increased glucose uptake, glycogen synthesis, and lipogenesis, and contributes to the mediation of the insulin–dependent suppression of hepatic glucose output (Choi and Kim, 2010). Hepatic AKT is essential in maintaining whole body glucose homeostasis and insulin responsiveness (Lu et al., 2012). Depletion of AKT from liver in mice causes insulin resistance and diabetic-like symptoms (Mackenzie and Elliott, 2014). In this sense, here we show that CR animals, but not CR-Leptin rats, displayed lower levels of hepatic AKT, in accordance with an impairment of insulin sensitivity, as suggested by their higher HOMA-IR score and the altered response at the gene expression level to WD feeding. Notably, leptin treatment during lactation partially restored AKT protein abundance in liver, which might be contributing to the

preservation of insulin sensitivity in these rats. Even though CR animals exhibited decreased AKT protein expression compared to controls, no significant differences were found concerning pAKT protein levels. This may be reflecting a greater activation of the AKT-dependent insulin signaling in CR animals, in accordance with the presence of higher basal insulin levels. In this regard, CR animals also showed a higher increase in pAKT levels in rWAT under WD conditions compared to control and CR-Leptin animals, while levels of total AKT did not present significant differences between groups. This may also be understood as the response of adipose tissue to higher basal insulin levels.

Concerning gene expression in rWAT, the increased expression levels of *Gk* in CR animals compared to control and CR-Leptin animals, particularly under WD conditions, may also reflect the presence of higher insulin levels. Insulin regulates glycerol kinase (GK) activity by enzyme induction, and increased activity of this protein has been observed in isolated fat cells of obese mice (Koschinsky et al., 1971). The physiological role of GK in fat cells is the phosphorylation of part of the glycerol generated during lipolysis; this may facilitate the esterification of fatty acids thus promoting fat deposition. This action has been described to have a small effect in lean animals, but it may be of major importance in obese ones (Koschinsky et al., 1971). On the other hand, the decrease of transcript levels of *Pparg* and *Pnpla2* in rWAT of SD-fed CR animals compared with controls, were also indicative of an alteration in energy metabolism towards lower lipogenesis and lipolysis capacity under these dietary conditions. Moreover, CR animals showed decreased expression levels of Mest versus control animals. Expression levels of this gene have been considered as a good marker of WAT expansion capacity, with low values being indicative of low adipose storage capacity (Nikonova et al., 2008; Voigt et al., 2013). Notably, expression patterns of the above-mentioned genes (*Gk*, *Pparg*, *Pnpla2*, *Mest*) were normalized in CR-Leptin animals; this could explain the normalization of the impaired capacity to handle lipid substrates, as well as to mobilize fat storage, and thus avoid the programmed predisposition to fat accumulation, which CR animals undergo in adulthood. CR-Leptin animals even showed higher expression levels of *Lpl* compared with control and CR animals, under both SD and WD conditions, suggesting increased capacity of fatty acid uptake from TG-rich lipoproteins. In fact, CR-Leptin animals showed lower circulating TG levels than those of CR animals and similar to those of controls. Therefore, CR animals may be less prepared for an efficient processing and handling of fuels, even under SD conditions, which is associated with an impaired lipid mobilization and clearance from circulation, and hence animals would be more likely to accumulate ectopic fat in tissues other than adipose tissue. In this regard, and in accordance with the insulin resistance features, WD-fed CR animals also showed signs of hepatic steatosis, which were more pronounced than in WD-fed control animals, along with increased expression of *Cd68* (a marker of macrophage infiltration) in WAT, even under SD. Thus, maternal undernutrition during gestation increases susceptibility to both hepatic steatosis and inflammation in adulthood, which may be more severe if animals are further exposed to nutritional challenges. Interestingly, the above-mentioned alterations were partially or

totally reversed in CR animals supplemented with leptin at physiological doses during the suckling period.

Besides insulin signaling, leptin sensitivity was also apparently impaired in the offspring of calorie-restricted animals during gestation, as evidenced by the presence of higher circulating leptin levels in adult CR animals compared to controls. Moreover, at the peripheral level, CR animals displayed lower expression levels of *Obrb* in WAT (under SD and WD) and in liver (under WD). The presence of peripheral leptin resistance in the offspring of calorie-restricted dams during gestation has been previously described (Palou et al., 2012). Unlike insulin resistance, leptin resistance associated with fetal undernutrition has been shown to appear in adulthood and under obesogenic conditions and seems to be secondary to insulin resistance or to central leptin resistance (Palou et al., 2012). The functionality of the leptin receptor in WAT is critical for the control of fat reserves. Leptin resistance at the adipose tissue levels has been associated with insulin resistance and dyslipidemia (Huan et al., 2003). The presence of diminished expression levels of leptin receptor in WAT of CR animals compared with controls may account, together with impaired response to insulin, for the decreased expression levels of *Pparg* and of *Pnpla2* that these animals exhibit under SD. Under WD conditions, the recovery of expression levels of both genes similar to those of controls could be tentatively related with the increase in *ObRb* expression occurring in these animals, as well as in the other groups, when exposed to this diet. It must be highlighted that expression levels of leptin receptor in WAT were totally normalized in CR-Leptin animals, which also showed the greatest increase in their expression levels under WD conditions. Leptin treatment during the suckling period in rat pups from normal nourished dams during gestation has also been shown to improve the lasting effects of a HF-diet on the abundance of leptin receptor in the adipose tissue and increase the oxidative capacity of these animals, resulting in a better handling of excess fuel (Priego et al., 2010).

Notably, although most alterations associated with undernutrition during gestation were reverted by leptin treatment during the suckling period, hepatic expression of the *ObRb* gene in CR-Leptin animals under WD was lower than that of controls and similar to that of CR animals. Leptin function in liver has not been clearly established; however, it has been shown to negatively regulate insulin action in this organ, having an important role modulating TG metabolism. Studies in mice with ablated hepatic leptin signaling have revealed that this loss can contribute to hepatic steatosis development, in agreement with the potent lipolytic effects of leptin, and results in larger, more triacylglyiceride-rich VLDL particles (Huynh et al., 2010; Huynh et al., 2013). In this sense, the presence of decreased expression levels of *ObRb* in WD-fed CR animals compared with controls could be related to the presence of increased lipid droplets in the liver and with higher plasma levels of TG. However, this argument does not explain the near absence of hepatic steatosis and the presence of plasma TG levels comparable to those of controls in WD-fed CR-Leptin animals, while having decreased *ObRb* expression levels. It could be hypothesized that decreased hepatic leptin signaling may not be enough to lead to relevant defects in lipid metabolism in absence of a general state of leptin and insulin

resistance. In fact, in mice, the effects of a loss in hepatic leptin signaling have been shown to be more pronounced in obese and hyperinsulinemic state (Huynh et al., 2013).

4.1.4. Conclusions

The present findings show that leptin supplementation at physiological doses during lactation improves energy homeostasis and metabolic control in adult animals jeopardized by undernutrition during gestation, and hence largely prevents the higher risk of accumulating excess fat and other disturbances of the metabolic syndrome, such as insulin resistance, hypertriglyceridemia, hepatic lipid deposition and adipose tissue inflammation. Therefore, this study brings additional evidence supporting the relevance of the intake of appropriate amounts of leptin throughout lactation to reverse postnatal sequelae induced by deficient fetal nutrition, and hence could be worth being considered in the search of strategies designed to treat and/or prevent the programmed trend to obesity.

4.2. CHAPTER 2

Leptin intake at physiological doses throughout lactation in rats restores the altered stomach sympathetic drive caused by mild gestational calorie restriction

Gestational undernutrition in rats has been associated with lower sympathetic innervation in offspring, affecting peripheral tissues such as the stomach. This has been linked to lower gastric secretion and decreased circulating levels of ghrelin. Considering the essential role of leptin intake during lactation to prevent obesity and reverse developmental malprogramming effects, and the implication in this function of the previously described capacity to be absorbed by the immature stomach in early postnatal life, we aimed to assess whether leptin supplementation reverses the decreased gastric sympathetic drive caused by mild gestational calorie restriction. Three groups of male rats were studied at a juvenile age and adulthood: 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 the density of TyrOH-immunoreactive ($TyrOH^+$) fibers, used as indicators of sympathetic innervation, were determined in the stomach and plasma leptin and ghrelin levels were determined. At 25 days, CR rats displayed lower density of $TyrOH^+$ fibers and lower TyrOH levels in the stomach than controls. These alterations were not found in CR-Leptin animals, suggesting that the altered sympathetic innervation was reverted by leptin treatment. This alteration was mitigated at the age of 6 months, and differences between control and CR animals were not significant. Nevertheless, the trend to a lower density of $TyrOH^+$ fibers in CR animals compared to controls was also reverted in CR-Leptin animals. Accordingly, CR animals, but not CR-Leptin ones, also showed lower serum ghrelin levels compared to controls, particularly at the age of 3 months, and a higher leptin/ghrelin ratio. These findings show that leptin intake during lactation is able to reverse the malprogrammed alterations in the stomach sympathetic drive and normalise the increased leptin/ghrelin ratio linked to a mild maternal calorie restriction during gestation in rats, supporting the relevance of leptin as an essential nutrient during lactation.

4.2.1. Background

An adverse perinatal environment is known to negatively affect the health of offspring in the long term (Gluckman et al., 2005; Godfrey and Barker, 2001; Picó et al., 2012). In this regard, there is epidemiological evidence linking undernutrition during foetal life, as well as low birth weight, and the major propensity to obesity and metabolic syndrome in adulthood (Gluckman et al., 2005; Godfrey and Barker, 2001; Ravelli et al., 1976). Several animal studies have also explored the effects of maternal undernutrition during gestation and have found that even a mild calorie restriction during the first period of gestation exerts detrimental effects in the offspring regarding energy balance and metabolic regulation, thus increasing the susceptibility to develop obesity-related pathologies in adulthood, especially when exposed to dietary stressful conditions (Palou et al., 2010a; Vickers et al., 2000). These programming effects and their potential reversion require special attention, particularly when considering strategies for obesity prevention from the early stages of life.

These detrimental effects have been attributed to an impairment in insulin and leptin sensitivity (Palou et al., 2012), together with defects on hypothalamic structure and function (Garcia et al., 2010), and alterations in the sympathetic innervation of white and brown adipose tissues (Garcia et al., 2011; Palou et al., 2015) and of stomach (Garcia et al., 2013), leading to a higher risk to develop hallmark features of the metabolic syndrome.

The decreased sympathetic innervation found in the stomach of pups submitted to mild calorie restriction during gestation was found to be associated with decreased blood ghrelin levels (Garcia et al., 2013). It is known that gastric sympathetic activation increases gastric ghrelin secretion, and noradrenaline released from sympathetic nerve terminals has been postulated as the principal factor involved in this process (Spencer et al., 2015). Ghrelin, together with leptin, are important appetite-regulating hormones. Unlike leptin, ghrelin, enhances appetite and increases food intake (Wren et al., 2001). However, circulating ghrelin has been reported to be decreased in obese subjects and inversely correlated with body mass index (Tschop et al., 2001). Moreover, low ghrelin concentrations have been associated with the prevalence of insulin resistance and type 2 diabetes (Poykko et al., 2003).

Besides the role of adipocitary leptin in body weight control, leptin is a natural component of breast milk (Casabiell et al., 1997), which is considered an essential factor during lactation in the protection against the development of obesity and related pathologies in later life (Palou and Pico, 2009; Pico et al., 2007; Sanchez et al., 2008). Notably, leptin supplementation at physiological doses throughout lactation has been shown to reverse some of the malprogrammed effects associated to mild calorie restriction during gestation, such as alterations in hypothalamic structure and function (Konieczna et al., 2013) and adipose tissue innervation (Konieczna et al., 2015a). However, whether leptin supplementation throughout lactation is able to reverse the aforementioned decrease in stomach sympathetic drive and its related alterations associated to gestational calorie restriction has not yet been explored.

In the present study we aimed to assess whether leptin supplementation throughout lactation in rats delivered from dams submitted to mild calorie restriction during gestation reverses the decreased sympathetic drive to the stomach and normalises circulating ghrelin levels.

4.2.2. Results

Study 1

Body weight and fat mass of animals at the age of 25 days are shown in Table 4.2.1. As previously described in the same cohort of animals (Konieczna et al., 2013), the offspring of calorie restricted dams during gestation showed lower body weight and body fat than controls ($P < 0.05$, LSD *post hoc* one-way ANOVA). In turn, leptin treatment throughout lactation had no demonstrable effects on the aforementioned parameters at this juvenile age.

Immunohistochemical analysis of TyrOH positive ($TyrOH^+$) fibers and western blood determination of specific TyrOH protein in the stomach of 25 day-old animals is shown in Figure 4.2.1. TyrOH is the rate-limiting enzyme for catecholamine biosynthesis, and its measure is usually used as an indicator of sympathetic innervation (Garcia et al., 2011). $TyrOH^+$ fibers were detected in the muscular layer area of the stomach. CR animals displayed significantly lower number of $TyrOH^+$ fibers compared to controls, whereas the alteration was totally reverted in CR-Leptin animals ($P < 0.05$, LSD *post hoc* one-way ANOVA). Analysis of specific TyrOH protein by western blot also revealed a significant decrease of TyrOH levels in the stomach of CR animals compared to controls, whereas CR-Leptin animals showed intermediate levels which were not different from control and CR animals ($P < 0.05$, LSD *post hoc* one-way ANOVA).

We therefore determined whether the decreased stomach sympathetic innervation occurring in CR animals affected circulating ghrelin levels (Table 4.2.1). In the present study, blood ghrelin levels in CR animals were slightly lower compared to control animals (25% decrease), but differences did not reach statistical significance. Notably, the trend to lower levels was completely reverted in CR-Leptin animals, which displayed levels very similar to controls. No significant differences between groups were found regarding ghrelin expression or protein levels in the gastric mucosa, and no alterations were observed in the weight of the stomach.

Circulating leptin levels are shown in Table 4.2.1. As previously described (Konieczna et al., 2013), CR animals displayed lower plasma leptin levels than controls, while CR-Leptin animals showed intermediate levels, which were not different from controls at the level of $P<0.05$ (LSD *post hoc* one-way ANOVA). No significant differences were found regarding the leptin/ghrelin (L/G) ratio.

	Control	CR	CR-Leptin	ANOVA
Juvenile age (25 days)				
Antropometric parameters				
Body weight (g)	65.5 ± 1.1^{a}	59.6 ± 1.2^{b}	60.2 ± 1.2^{b}	T
Fat mass (%)	7.37 ± 0.28 ^{a}	5.56 ± 0.20 ^{b}	5.87 ± 0.22 ^{b}	T
Circulating parameters				
Leptin (µg/L)	2.21 ± 0.17^{a}	1.79 ± 0.12 ^{b}	2.04 ± 0.05 ^{ab}	T
Ghrelin (µg/L)	2.20 ± 0.28	1.66 ± 0.30	2.26 ± 0.67	
Leptin/ghrelin	1.11 ± 0.20	0.91 ± 0.35	1.45 ± 0.66	
Stomach parameters				
Stomach weight (mg)	478 ± 12	513 ± 17	481 ± 18	
Ghrelin mRNA (Au)	100 ± 7	80 ± 8	76 ± 6	
Ghrelin levels (ng/g)	221 ± 24	225 ± 18	239 ± 18	

Table 4.2.1. Anthropometric, circulating and stomach parameters of animals at the age of 25 days. Data are mean ± s.e.m. Circulating leptin and ghrelin were determined under feeding conditions. Statistics related-symbols: T, effect of early treatment (C, CR and CR-Leptin) ($P < 0.05$, one-way ANOVA). Data not sharing a common letter (a, b) are significantly different.

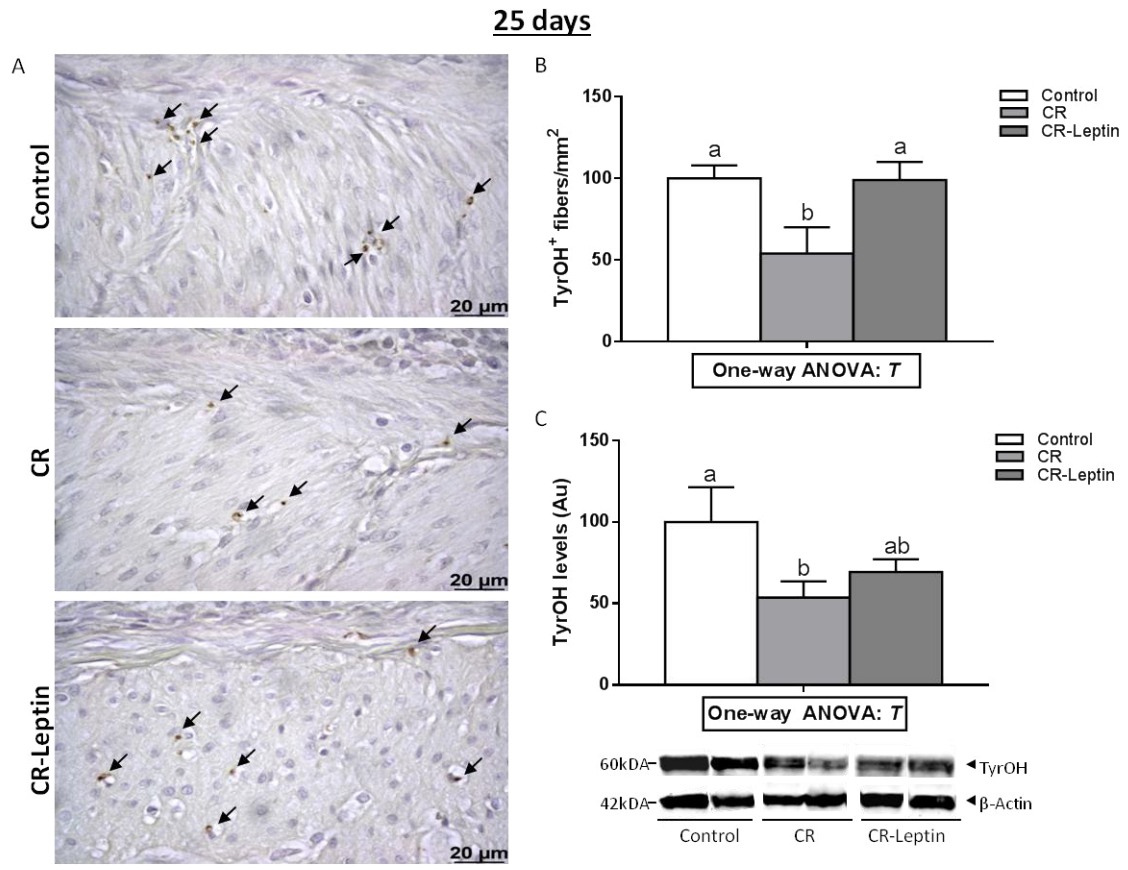


Figure 4.2.1. Immunohistochemical analysis of TyrOH positive ($TyrOH^+$) fibers and western blot determination of specific TyrOH protein in the stomach of control, CR and CR-Leptin animals at the age of 25 days. A) Representative immunostained sections for TyrOH (brown colour), indicated by black head arrows in the muscular layer area. Scale bar: 20 µm. B) Number of $TyrOH^+$ (fibers/mm^2 of stomach). C) Specific protein levels of TyrOH in the stomach expressed relative to levels of β-Actin (loading control). Representative bands for TyrOH and β-Actin are shown. 150 ug of protein were loaded in each lane. Results represent mean ± s.e.m. (n=10-11). Statistics related-symbols: T, effect of early treatment (C, CR and CR-Leptin) ($P < 0.05$, one-way ANOVA). Data not sharing a common letter (a, b) are significantly different.

Study 2

A new cohort of animals was followed up until 6 months of age with the purpose of determining whether the beneficial effects of leptin treatment during lactation allowing the recovery of malprogramming effects of gestational calorie restriction on the stomach sympathetic innervation found at a juvenile age were maintained in adulthood.

Detailed phenotypic characterization and blood parameters of the same cohort of animals from 21 days to 6 months have been included in chapter 1 (Table 4.1.1). Some of the data relating to body weight parameters and cumulative food intake are summarized in Table 4.2.2, including body fat percentage at 6 months. No differences between groups were found regarding body weight, body fat and cumulative food intake. Notably, despite no significant differences, CR animals showed a trend to greater adiposity than control, whereas CR-Leptin animals showed intermediate levels.

$TyrOH^+$ fibers were also determined by immunohistochemical analysis in the stomach of 6 month-old animals (Figure 4.2.2). This measurement was performed in the inner oblique muscular layer, since it was the most comparable layer between the different animals. Results showed that changes observed regarding the number of $TyrOH^+$ fibers between control and CR animals at a juvenile age appear to weaken in adulthood. More specifically, CR animals displayed a tendency to have a lower number of $TyrOH^+$ fibers compared to controls, although differences were not significant. Notably, the beneficial effects of leptin treatment during lactation found at early ages were maintained in adulthood, since CR-Leptin animals maintained a number of $TyrOH^+$ fibers comparable to controls. Similar results were also observed for the measurement of specific TyrOH levels in the stomach by western blot, although as occurring with the number of $TyrOH^+$ fibers, no significant differences were found between groups (Figure 4.2.2).

Regarding circulating ghrelin, at 3 months of age, CR-Leptin animals presented greater fasting ghrelin levels than CR animals, whereas CR animals displayed a trend to lower levels than controls ($P < 0.05$, LSD *post hoc* one-way ANOVA) (Table 4.2.2). The same trend was found at the age of 6 months, but differences between groups were not significant. No significant differences between groups were found considering data at 3 and 6 months, but levels at 6 months were significantly lower than at 3 months ($P < 0.05$, repeated measures ANOVA). According to the results found at a juvenile age, no significant differences between groups were found regarding ghrelin expression or the weight of the stomach at the age of 6 months.

No significant differences were found between groups concerning fasting circulating leptin levels in adult rats (Table 4.2.2). However, CR animals, but not CR-Leptin animals, displayed a greater L/G ratio than controls ($P < 0.05$, repeated measures ANOVA). Animals at 6 months of age showed a higher L/G ratio compared to values at 3 months ($P < 0.05$, repeated measures ANOVA), but no differences were found between groups.

		Control	CR	CR-Leptin	ANOVA
Adulthood					
Antropometric parameters and food intake					
Body weight (g)	3 m	355 ± 8	370 ± 9	363 ± 7	
	6 m	442 ± 21	461 ± 20	442 ± 18	
Fat mass (%) (6 m)		16.5 ± 1.1	18.8 ± 0.9	18.2 ± 1.0	
Cumulative food intake (Kcal) (21d-6m)		11731 ± 153	11326 ± 163	11415 ± 165	
Circulating parameters					
Leptin (µg/L)	3 m	1.81 ± 0.27	2.99 ± 0.46	2.84 ± 0.37	
	6 m	2.76 ± 0.34	3.78 ± 0.85	2.68 ± 0.33	
Ghrelin (µg/L)	3 m	21.3 ± 1.1^{ab}	18.7 ± 0.6^{a}	21.7 ± 1.1^{b}	Age
	6 m	15.2 ± 0.7*	14.4 ± 0.9*	15.8 ± 1.4*	
Leptin/ghrelin	3 m	0.09 ± 0.02 A	0.18 ± 0.04 B	0.13 ± 0.02 A	T, Age
	6 m	0.16. ± 0.02*	0.27 ± 0.06	0.17± 0.03*	
Stomach parameters (6 m)					
Stomach weight (mg)		2036 ± 65	2194 ± 102	2194 ± 86	
Ghrelin mRNA (Au)		100 ± 17	115 ± 18	113 ± 25	

Table 4.2.2. Anthropometric characteristics, food intake, circulating and stomach parameters of adult animals. Data are mean ± s.e.m. Circulating leptin and ghrelin levels were determined under fasting conditions. Repeated measures analysis of variance (ANOVA) was carried out to assess the effects of perinatal treatment (controls, CR, CR-Leptin) and the age of animals (3 or 6 months). One-way ANOVA was used to determine differences between groups at the ages of 3 and 6 months separately, when no significant differences were found considering both ages together, and for data obtained only at the age of 6 months. This was followed by a least significance difference (LSD) *post hoc* test. Single comparisons between groups were assessed by Student's *t*-test. Statistics related-symbols: T, effect of early treatment (C, CR and CR-Leptin); Age, effect of age (6 months versus 3 months) ($P < 0.05$, Repeated measures ANOVA). Data not sharing a common letter (A, B for repeated measures ANOVA or a, b for one-way ANOVA are significantly different). *, different from their respective value at 3 months of age (Student's *t*-test).

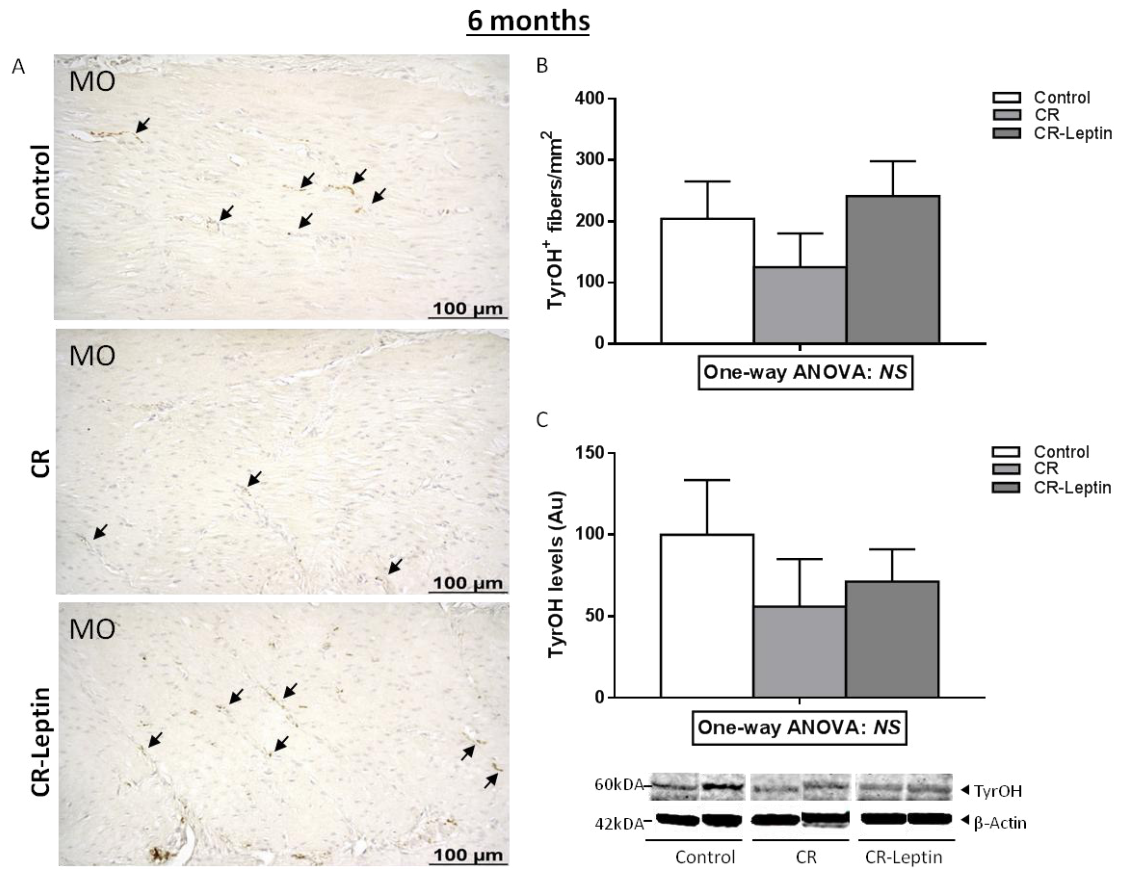


Figure 4.2.2. Immunohistochemical analysis of TyrOH positive ($TyrOH^+$) fibers and western blot determination of specific TyrOH protein in the stomach of control, CR and CR-Leptin animals at the age of 6 months. A) Representative immunostained sections for TyrOH (brown colour), indicated by black head arrows, in the inner oblique muscular layer of the stomach. Scale bar: 100 µm. B) Number of $TyrOH^+$ (fibers/mm^2 of stomach). C) Specific protein levels of TyrOH in the stomach expressed relative to levels of β-Actin (loading control). Representative bands for TyrOH and β-Actin are shown. 150 ug of protein were loaded in each lane. Results represent mean ± s.e.m. (n=7-9). Symbols: *NS*: no significant differences by one-way ANOVA. MO: muscular oblique layer.

4.2.3. Discussion

Calorie restriction during gestation has been reported to trigger detrimental effects in the offspring by causing a number of metabolic disturbances and affecting the capacity to properly regulate energy balance in adulthood (Picó et al., 2012; Vickers et al., 2000). Leptin supplementation at physiological doses throughout lactation has been shown to revert, at least in part, most of the malprogramming effects in the offspring associated to a mild calorie restriction during gestation (Konieczna et al., 2013; Konieczna et al., 2015a), hence normalising to a greater extent the phenotypic alterations observed in adulthood (chapter 1).

Among the mechanisms responsible for phenotypic alterations in the offspring of calorie restricted dams during gestation, we have previously reported the presence of a decreased sympathetic drive to the stomach (Garcia et al., 2013). This alteration was proposed as the responsible cause, or at least one of the contributing factors, for the presence of reduced circulating ghrelin levels in these animals, according to the

involvement of sympathetic stimulation on gastric ghrelin secretion (Garcia et al., 2010; Garcia et al., 2011; Garcia et al., 2013). It should be mentioned that these alterations were found at early ages, before the phenotypic changes described in adult animals became evident. The present study has confirmed the presence of a reduced sympathetic drive in the stomach of 25 day-old pups born from dams submitted to a calorie restriction during gestation. Moreover, we present the first evidence here that this alteration was reversed in pups treated with physiological doses of orally taken leptin throughout lactation. Sympathetic innervation was determined by assessing TyrOH, using both biochemical (protein content) and morphological (tissue immunostaining) approaches, methods, previously used to estimate sympathetic innervation in the adipose tissue and in the stomach (Garcia et al., 2013; Giordano et al., 2005). The alteration in sympathetic drive found at a juvenile age was less marked and not significant at the age of 6 months; however, the positive effects of leptin supplementation were still evident.

Further benefits of leptin treatment throughout lactation in the offspring of calorie-restricted dams during gestation were also observed when looking at circulating ghrelin levels. Ghrelin is an important hormone mainly secreted by the stomach that acts in the brain to regulate food intake, body weight, adiposity and glucose metabolism (Tschop et al., 2000). Leptin treatment during the suckling period resulted in higher ghrelin levels and restored the diminished levels found in CR animals, although differences between groups were only significant at the age of 3 months. In line with previous findings (Garcia et al., 2013), this study supports that the decreased ghrelin levels found in CR animals do not seem to be related to lower ghrelin production by the stomach, because no differences were found between groups concerning their mRNA expression levels, either at the age of 25 days or at 6 months. Rather, they seem to be related to lower gastric ghrelin secretion, in accordance with the decreased sympathetic drive to the stomach. Nevertheless, it is worthy to mention that, although alterations in circulating ghrelin levels appear to be due to disturbances in the sympathetic drive to the stomach, the influence of other factors cannot be ruled out.

The presence of decreased ghrelin levels in CR rats, which are known to increase the susceptibility to develop obesity in adulthood, could seem controversial when considering the orexigenic actions of ghrelin (Kamegai et al., 2001; Tschop et al., 2000; Wren et al., 2001). However, endogenous ghrelin does not appear to be an essential regulator of food intake, as demonstrated in ghrelin-null mice, which do not exhibit appreciable alterations in the regulation of food intake or body weight (Wortley et al., 2004). Moreover, plasma ghrelin levels have been reported to be reduced in animal models of obesity (Ariyasu et al., 2002; Levin et al., 2003) and in obese subjects, being inversely correlated with adiposity, fasting insulin, and leptin (Tschop et al., 2001). In this regard, we have found no differences in food intake between control and CR animals, but CR animals are known to have a greater tendency towards excess fat accumulation along with higher insulin levels and HOMA-IR index, particularly when exposed to obesogenic diets (chapter 1). In turn, normalisation of ghrelin levels in CR-

Leptin animals might account for the reversion of the above mentioned alterations, as previously described in chapter 1.

Leptin has a well-known role in the regulation of energy balance and is also considered to play a key role in the pathogenesis of obesity-related disorders. Besides the relevance of fasting levels of leptin and ghrelin individually, the L/G ratio may be more sensitive to predict alterations related with components of the metabolic syndrome, like obesity and insulin resistance. In fact, this ratio has been described to decrease in type 2 diabetes patients who experienced an improvement in insulin sensitivity (Hajimohammadi et al., 2017). Moreover, a low L/G ratio has been considered a good marker to predict better metabolic adaptation and increased weight loss after dietary intervention in obese women (Labayen et al., 2011) and successful weight maintenance after dietary intervention in obese subjects (Crujeiras et al., 2014; Crujeiras et al., 2010). In this sense, we found here that adult CR animals displayed significantly greater values of the L/G ratio than controls, in accordance to the increased tendency of these animals to develop metabolic syndrome characteristics, particularly when exposed to an obesogenic diet (chapter 1). Notably, this ratio was completely normalised in CR animals treated with oral leptin throughout lactation. These findings, besides showing reversion of altered malprogramming structures and functions due to adverse prenatal conditions, are in favour of the potential usefulness of the L/G ratio measurement in adulthood as a clinical non-invasive marker of programmed susceptibility to develop obesity-related alterations; furthermore, it also seems to be able to detect the reversion of these disruptions by early life treatment, being more sensitive than leptin or ghrelin alone.

4.2.4. Conclusions

The present results show for the first time that treatment with physiological doses of oral leptin throughout lactation to the offspring of gestational calorie-restricted dams reverses the decreased sympathetic innervation in the stomach associated to maternal undernutrition. Normalisation of the stomach sympathetic drive during lactation may contribute to the normalisation of circulating ghrelin levels and particularly of the L/G ratio in adulthood. These findings give further support to the benefits of the presence of leptin in breast milk compared to infant formulae, which lacks leptin.

4.3. CHAPTER 3

Identification of blood cell transcriptome-based biomarkers in adulthood predictive of increased risk to develop metabolic disorders due to gestational undernutrition and sensitive to its reversion by leptin supplementation

Mild calorie restriction during gestation has long-lasting effects in the offspring inducing metabolic syndrome-related alterations, which might be predicted by gene expression profiling in PBMCs during the first month of life in rat and can be beneficially reverted by oral supplementation with leptin during lactation. However, whether or not PBMCs gene-expression profiling in adulthood can be used to predict later metabolic alterations has not been previously investigated. In this chapter we used this animal model to determine potential transcript-based biomarkers in PBMCs in adulthood indicative of a higher risk to develop obesity-related metabolic alterations and sensitive to early nutritional intervention. Gene expression microarray analysis was performed in PBMCs from adult male Wistar rats belonging to three experimental groups: the offspring of control dams fed ad libitum with standard chow diet during gestation (controls); the offspring of 20% calorie-restricted dams from day 1 to 12 of gestation (CR); and CR rats supplemented with a daily oral dose of leptin throughout lactation (CR-Leptin). A group of 401 unique transcripts were affected by gestational calorie restriction and reverted by leptin treatment throughout lactation. Such alterations were found before phenotypic changes related to the metabolic syndrome were manifested. Among the genes identified, two were associated to feeding control, *Agrp* and *Pomc*, and others with lipid metabolism and with clear connections to insulin homeostasis, hepatic steatosis, and cardiovascular health, such as *Pcyt2*, *Rxrβ*, *Insig1*, *Npc1*, *Slc27a4*, *Apoo*, *Pla2g2a,* and *Sort1*. Functional microarray analysis of PBMCs of animal models that underwent prenatal and early postnatal interventions revealed a set of potential blood-cell transcript-based biomarkers in adulthood which, according to existing biological knowledge, may be indicative of predisposition to alterations related to the metabolic syndrome and sensitive to its reversion by early life nutritional intervention.

4.3.1. Background

Prevention of chronic diseases, particularly obesity and its related alterations, is one of the major challenges in developed societies (Malik et al., 2013a). Diagnosis of the risk to these diseases with appropriate biomarkers may help its prevention and management. However, there is a lack of reliable biomarkers of predisposition to these conditions, and hence the identification of new biomarkers could help to implement preventive strategies and decrease their prevalence (Musaad and Haynes, 2007). Numerous studies carried out in the last decade have successfully used peripheral blood cells, either the sub-fraction of mononuclear cells (peripheral blood mononuclear cells, PBMCs) or whole blood cells, as a source of candidate transcriptomic biomarkers of health and disease (Oliver et al., 2013; Sanchez et al., 2014; Sanchez et al., 2012; Takamura et al., 2007). The interest of these cells as a surrogate tissue lies in that blood samples can be obtained in sufficient quantities with minimally invasive techniques compared with other tissue samples (de Mello et al., 2012). Moreover, their gene expression profile reflects in part the expression profile that occurs in other tissues, such as liver and adipose tissue, and hence may reflect the physiological and pathological state of the body (Konieczna et al., 2014; Oliver et al., 2013).

The phenotypes of health or disease in adulthood are the result of the interaction between genetic and environmental factors, but metabolic programming during the fetal and perinatal period of development also plays a key role (Gluckman et al., 2005; Hales and Barker, 2001). In fact, a growing body of evidence suggests that maternal nutritional conditions during critical stages of life result in long term consequences on the future metabolic health of the offspring, affecting their propensity to obesity in adulthood (Gluckman et al., 2005; Hales and Barker, 2001; Picó et al., 2012). In this context, human and animal models associate gestational undernutrition with adverse health outcomes in adult offspring (Kiani A, 2011; Picó et al., 2012; Vickers et al., 2000). Mechanisms underlying the dysregulation of energy homeostasis due to perinatal nutrition have been associated, among other processes, to a lower capacity to respond to insulin and leptin signaling (Palou et al., 2012; Palou et al., 2010a), a perturbed hypothalamic structure and function (Garcia et al., 2010; Ikenasio-Thorpe et al., 2007), and a reduced innervation and functionality of white and brown adipose tissues (Garcia et al., 2011; Palou et al., 2015) and of the stomach (Garcia et al., 2013).

Hormones such as glucocorticoids, insulin and leptin are the main candidates as early life causative factors involved in developmental programming (Kiani A, 2011). Furthermore, leptin, a natural component of breast milk (Casabiell et al., 1997), is considered an essential factor during lactation in the protection against the development of obesity and related pathologies in later life (Palou and Pico, 2009). Leptin supplementation at physiological doses throughout lactation to rat pups protects the offspring against the development of age- and high-fat diet related excess body weight and other metabolic alterations (Pico et al., 2007; Sanchez et al., 2008). Moreover, dietary leptin supplementation to the offspring of calorie-restricted rats during gestation reverses part of the malprogrammed effects associated to undernutrition during gestation identified at early ages, including alterations in hypothalamic structure and

function (Konieczna et al., 2013) and adipose tissue innervation (Konieczna et al., 2015a), and normalizes to a large extent the altered gene expression profile in PBMCs found at early ages associated with undernutrition during pregnancy (Konieczna et al., 2015b).

The beneficial effects of leptin treatment during lactation preventing the detrimental effects of gestational undernutrition have been described to be phenotypically evident in adulthood, particularly in rats exposed to an obesogenic diet (chapter 1). Leptin supplementation largely prevents the higher risk of accumulating excess fat and other disturbances of the metabolic syndrome, such as insulin resistance, hypertriglyceridemia and hepatic lipid deposition (chapter 1). Therefore, considering the interest in the identification of biomarkers for obesity prevention, the above mentioned animal models, which presented a bias due to the influence of environmental and nutritional factors at early ages (i.e. rats exposed to mild undernutrition during gestation and rats treated with leptin during lactation), may be useful in adulthood, but prior to the manifestation of phenotypic alterations , for the identification of biomarkers predictive of increased risk to metabolic disorders and its reversion by intervention in early life.

In the present study we performed whole genome transcriptome analysis of PBMCs samples from the adult offspring of control dams and of calorie-restricted dams during gestation (CR) and of CR rats supplemented with dietary leptin (CR-Leptin) during the suckling period to identify novel potential biomarkers in adult age indicative of a higher risk to develop alterations related to the metabolic syndrome and also sensitive to its reversion by nutritional intervention during early life.

4.3.2. Results

Phenotypic characteristics and circulating parameters

Body weight and fat mass, both at weaning and at the age of 4 months were not significant different between control, CR, and CR-Leptin animals (Table 4.3.1). Also, no significant differences between groups were found concerning circulating glucose, leptin and insulin levels, as well as the HOMA-IR index, at the age of three months (Table 4.3.1).

a. Anthropometric measurements	Control	CR	CR-Leptin
Body weight, 21 days (g)	44.3 ± 1.8	45.9 ± 0.6	46.1 ± 0.7
Body weight, 4 months (g)	399 ± 10	409 ± 10	402 ± 8
Fat mass 21days (g)	4.2 ± 0.3	4.5 ± 0.1	4.4 ± 0.2
(%)	9.4 ± 0.3	9.7 ± 0.3	9.3 ± 0.5
Fat mass 4 months (g)	56.9 ± 3.2	65.1 ± 3.8	60.4 ± 3.2
(%)	14.3 ± 0.7	15.9 ± 0.8	14.9 ± 0.6
b. Circulating parameters			
Glucose (mg/dl)	96 ± 6	96 ± 4	97 ± 3
Leptin (µg/l)	1.8 ± 0.3	3.0 ± 0.5	3.0 ± 0.4
Insulin (µg/l)	0.5 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
HOMA-IR index	2.1 ± 0.4	3.1 ± 0.4	2.6 ± 0.4

Table 4.3.1. Anthropometric and circulating parameters. Controls: the offspring of control dams fed ad libitum with standard chow diet during gestation; CR: the offspring of 20% calorie-restricted dams during gestation; and CR-Leptin: CR rats supplemented with a daily oral of leptin throughout lactation. Circulating parameters were measured at the age of three months under fasting conditions. Data are mean ± SEM. No significant differences were found by one way ANOVA ($P > 0.05$).

Gene expression in PBMCs at the age of 4 months based on whole-genome microarray analysis

Whole genome microarray analysis was performed in PBMCs of 4-month-old animals from control, CR and CR-Leptin groups. In our microarray analysis, 59.2% of probes appeared to be expressed and were analyzed further. In total, 3,043 probes were found to be significantly different between control and CR animals ($P < 0.05$; Student's t-test). Subsequently, considering this group of probes, statistical analyses (Student's t-test) were performed to compare expression levels between CR and CR-Leptin groups and between CR-Leptin and control groups. In total, 871 probes were found to be significantly different between control and CR animals and simultaneously not statistically different between control and CR-Leptin animals ($P < 0.05$; Student t-test), so the expression levels of these genes were totally or partially reverted by oral leptin treatment throughout lactation. After duplicate removal, they represent 484 unique genes, of which 83 genes were unknown ones, and therefore were not taken into account for the subsequent classification nor for the PCA or heat map representations.

The 401 remaining genes (referred as "core group" of genes) were classified into several biological processes according to their function, using the databases described in the Materials and Methods section. Among the known genes, 178 exhibited down-regulation and 223 up-regulation (Figure 4.3.1). As shown in Figure 4.3.1, the processes with the highest number of differentially expressed genes were related to transcription and translation machinery, cell turnover, transport, signaling and protein metabolism. Additional processes with a remarkable number of genes were related to immune system, cytoskeleton and lipid metabolism. The remaining genes were related to neuronal signaling, carbohydrate metabolism, cell communication, vascular homeostasis, nervous system, redox metabolism, inositol metabolism, nucleic acid and

nucleotide metabolism, vitamin and mineral metabolism, central metabolism, amino acid and polyamines metabolism, feeding control, blood and miscellanea (see detailed list in **Section 8.2, Annex II**). Detailed description of genes related to feeding control and lipid metabolism is shown in Table 4.3.2.

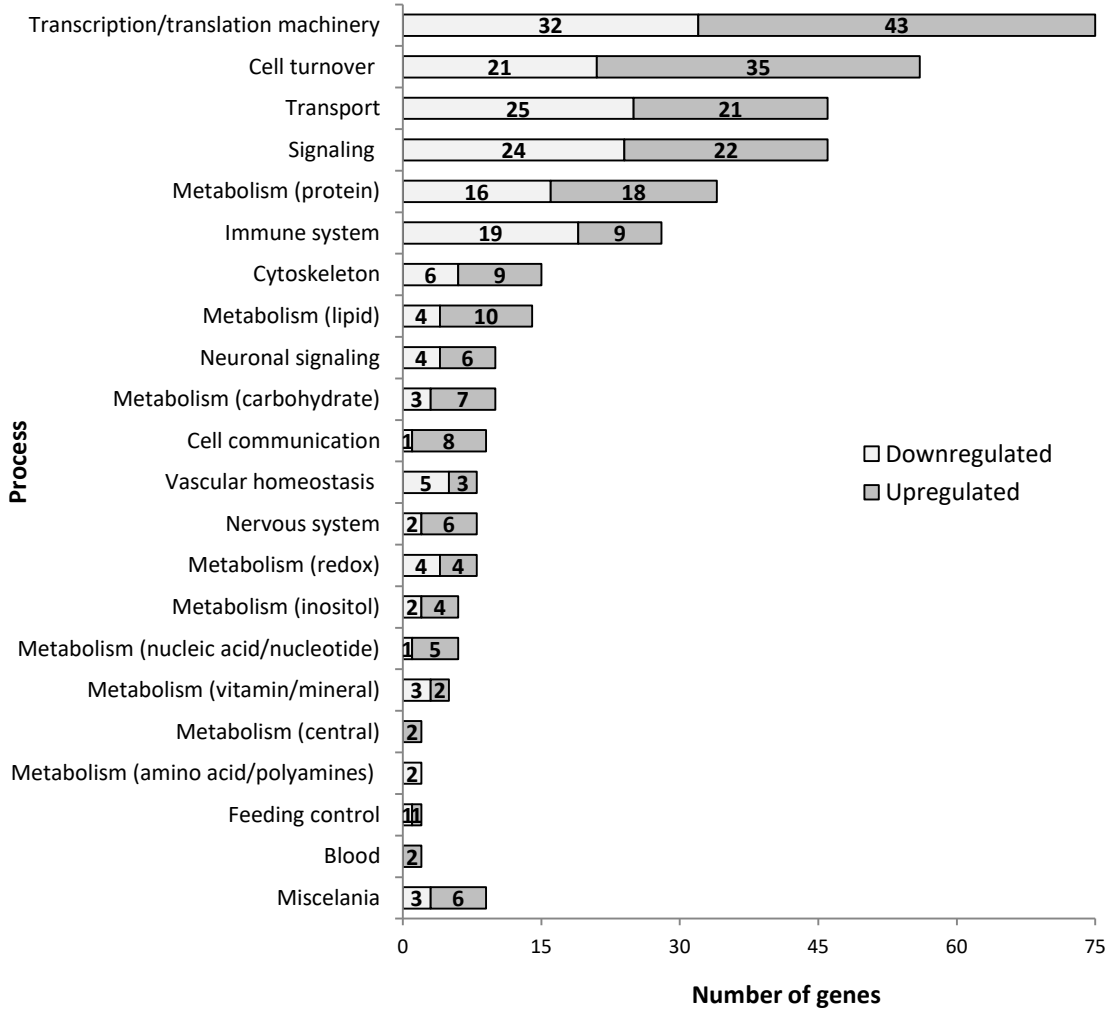


Figure 4.3.1. Microarray data classification into biological processes of the core group of 401 known genes differentially expressed in PBMC samples between control and CR animals and simultaneously not different between control and CR-Leptin animals at the age of 4 months. Statistical analysis between groups was performed ($P < 0.05$, Student´s *t*-test). The number of genes down- or up- regulated is indicated for each group of genes.

Of the 401 known unique genes differently expressed in CR vs control animals and simultaneously not different between control and CR-Leptin animals, nine were identified to be also significantly different between CR and CR-Leptin groups ($P < 0.05$; Student's *t*-test) and at the same time not statistically different between control and CR-Leptin animals. These nine unique genes thus show total reversion in their differential expression upon oral leptin treatment throughout lactation, and they are: centromere protein A (*Cenpa*), clathrin, light chain A (*Clta*), F-box protein 31 (*Fbxo31*), hemoglobin, epsilon 1 (*Hbe1*), MAX network transcriptional repressor (*Mnt*),

neurofibromin 1 (*Nf1*), plasminogen activator, urokinase receptor (*Plaur*), rennin (*Ren*), and regulator of microtubule dynamics 3 (*Rmdn3*). The remaining 392 genes include the set of genes whose expression levels differed significantly between control and CR animals but did not between CR-Leptin and CR animals and between CR-Leptin and control animals, thus a partial revertion when the pups were treated with leptin throughout lactation.

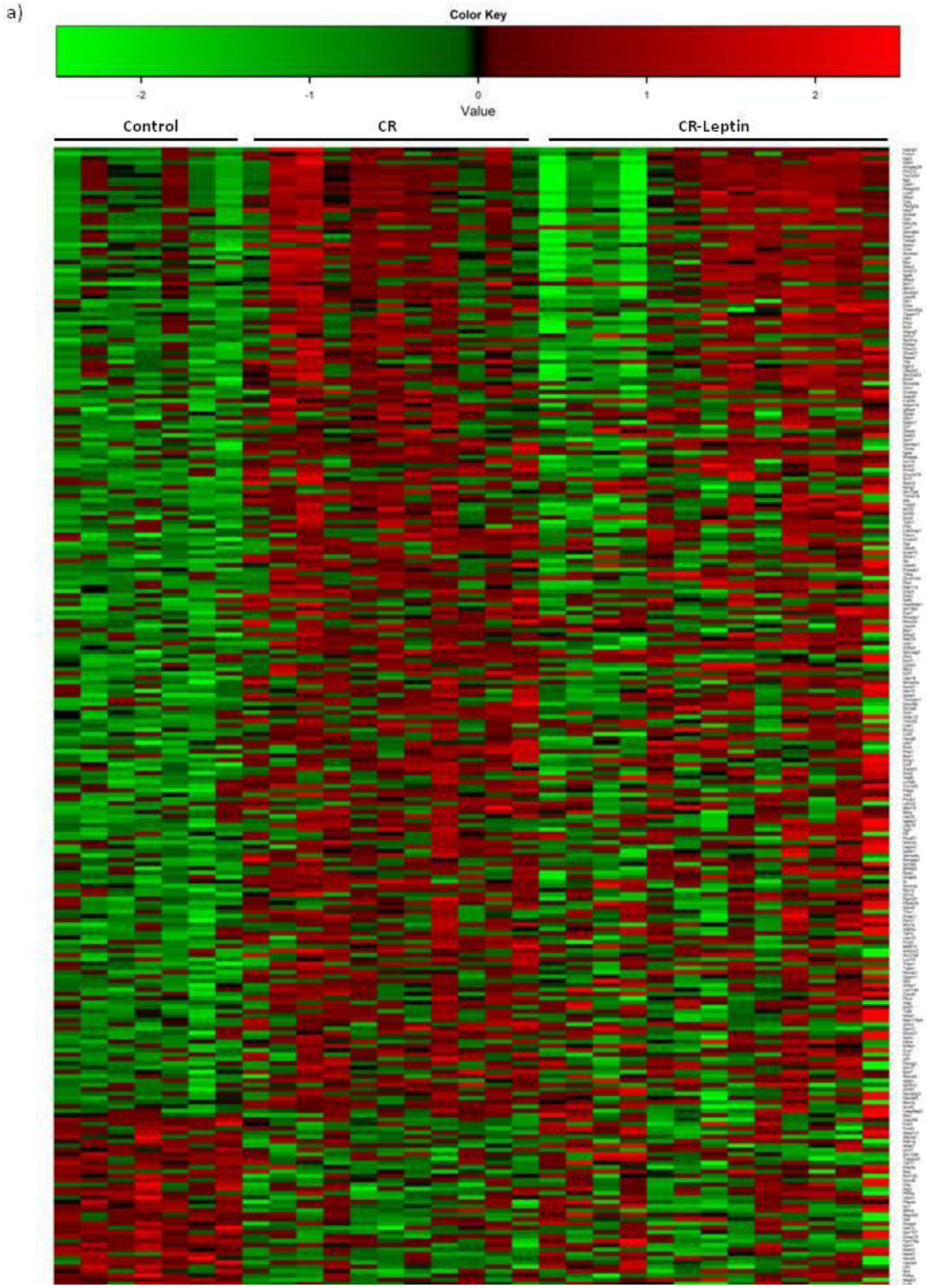
The results of the microarray analysis concerning the core group of 401 genes across individual samples are depicted in a heat map (Figure 4.3.2a). This figure illustrates the main differences in gene expression levels between control and CR groups, and how these differences became partially or even fully reverted in CR-Leptin animals.

The principal components analysis (PCA) performed with the core group of 401 genes (Figure 4.3.2b) also illustrates the different expression pattern of CR animals versus control animals, which were mainly differentiated across principal component 1, whereas CR-Leptin animals were spread through the control and CR groups.

Biological process	Gene symbol	Gene name	Sequence ID	Microarray					
				CR vs Control		CR-Leptin vs CR		CR-Leptin vs Control	
				P	FC	*P*	FC	*P*	FC
Feeding control	*Agrp*	Agouti related neuropeptide	NM_033650	0.020	1.71	0.180	0.70	0.518	1.20
	Pomc	Proopiomelanocortin	NM_139326	0.005	0.88	0.507	1.04	0.121	0.91
Metabolism (lipids)	*Acad10*	Acyl-CoA dehydrogenase family, member 10	NM_001134537	0.005	1.17	0.845	0.99	0.062	1.15
	Acot2	Acyl-CoA thioesterase 2	NM_138907	0.032	1.06	0.339	0.96	0.624	1.02
	Apoo	Apolipoprotein O	NM_001199178	0.028	1.09	0.521	0.97	0.278	1.05
	Dgkg	Diacylglycerol kinase, gamma	NM_013126	0.032	0.89	0.339	1.06	0.283	0.94
	Insig1	Insulin induced gene 1	NM_022392	0.022	0.78	0.459	1.07	0.084	0.83
	Liph	Lipase, member H	NM_001044279	0.046	1.37	0.162	0.79	0.667	1.08
	Npc1	Niemann-Pick disease, type C1	NM_153624	0.003	0.84	0.546	1.04	0.066	0.87
	Pcyt2	Phosphate cytidylyltransferase 2, ethanolamine	NM_053568	0.014	1.10	0.354	0.97	0.108	1.07
	Pla2g2a	Phospholipase A2, group IIA (platelets, synovial fluid)	NM_031598	0.042	1.51	0.306	0.73	0.769	1.10
	Rxrβ	Retinoid X receptor beta	NM_206849	0.037	1.13	0.072	0.91	0.687	1.02
	Sar1a	Secretion associated, Ras related GTPase 1A	NM_001007739	0.016	0.88	0.407	1.03	0.071	0.91
	Slc27a4	Solute carrier family 27 (fatty acid transporter), member 4	NM_001100706	0.044	1.10	0.416	0.97	0.177	1.07
	Sort1	Sortilin 1	NM_031767	0.014	1.19	0.698	0.96	0.192	1.15
	Tm7sf7	Transmembrane 7 superfamily member 2	NM_001013071	0.038	1.12	0.309	0.95	0.217	1.07

Table 4.3.2. Detailed list of genes related to feeding control and lipid metabolism within the core group of 401 known genes differentially expressed in PBMC samples between control and CR animals and simultaneously not different between control and CR-Leptin animals at the age of 4 months. Controls: the offspring of control dams fed ad libitum with standard chow diet during gestation; CR: the offspring of 20% calorie-restricted dams during gestation; and CR-Leptin: CR rats supplemented with a daily oral of

leptin throughout lactation. *P*-values (*P*) of microarray data (Student's *t*-test) and fold change (FC) values of CR vs Controls, CR-Leptin vs CR and CR-Leptin vs Control comparisons are indicated; FC > 1 indicates up-regulation; FC < 1 indicates down-regulation.



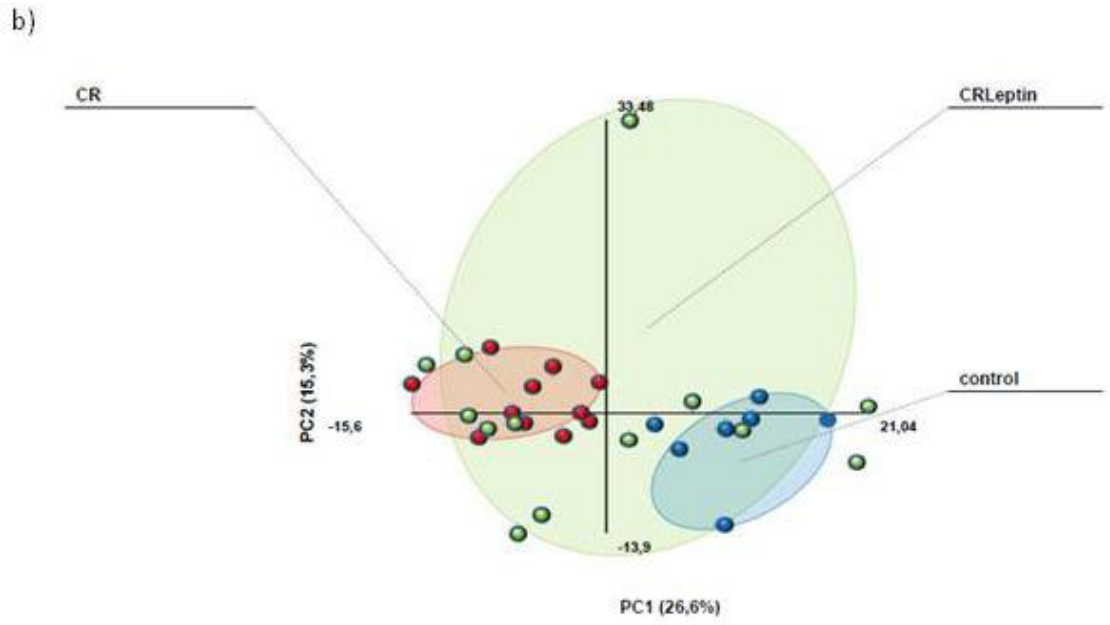


Figure 4.3.2. a) Heat map representing individual expression data of the core group of 401 known genes differentially expressed in PBMC samples between control and CR animals and simultaneously not different between control and CR-Leptin animals at the age of 4 months. Rows represent the genes sorted by fold change and the columns represent the value of the expression of each gene after a range scaling method (zero-mean centered) in each animal; **b)** Principal components analysis (PCA) plot with expression data of the core group of genes. Control, CR and CR-Leptin animals are plotted based on their loading scores on the first two principal components in blue, red and green colors, respectively. Confidence ellipses are drawn around each group. PCA analysis was performed using the Excel add-in Multibase package (Numerical Dynamics, Japan). Controls: the offspring of control dams fed ad libitum with standard chow diet during gestation; CR: the offspring of 20% calorie-restricted dams during gestation; and CR-Leptin: CR rats supplemented with a daily oral of leptin throughout lactation.

Confirmation and verification of microarray results by RT-qPCR

RT-qPCR analysis of a selected group of genes (*Agrp*, *Pomc*, *Il1b* and *Il10*) was performed in PBMCs from the three groups of animals (Table 4.3.3a and 4.3.3b). In the case of *Pomc*, *Il1b* and *Il10*, transcript levels in CR animals were significantly lower than in control animals, while in the case of *Agrp*, transcript levels were significantly higher ($P < 0.05$, one-way ANOVA). In all cases, CR-Leptin animals showed intermediate expression levels not significantly different from control and CR animals. This expression pattern is the same as the one observed in the microarray analysis (Table 4.3.3a).

Gene Simbol	Experimental group			Sequence ID	Microarray					
					CR vs Control		CR-Leptin vs CR		CR-Leptin vs Control	
	Control	CR	CR-Leptin		*P*	FC	*P*	FC	*P*	FC
Agrp	100 ± 16	194 ± 30	139 ± 27	NM_033650	0.020	1.71	0.180	0.70	0.518	1.20
Il1b	100 ± 12	53 ± 7	80 ± 14	NM_031512	0.002	0.54	0.433	1.22	0.115	0.66
IL10	100 ± 21	42 ± 8	70 ± 13	NM_012854	0.027	0.8	0.418	1.06	0.119	0.85
Pomc	100 ± 7	66 ± 6	82 ± 5	NM_139326	0.005	0.88	0.507	1.04	0.121	0.91

Table 4.3.3a. List of the expression of selected genes (%), sequence ID, *p*-value and fold change used in qRT-PCR to confirm the microarray analysis. Abbreviations: Agouti-related peptide (*Agrp*), interleukin 1 beta (*Il1b*), interleukin 10 (*Il10*), proopiomelanocortin (*Pomc*). *P*-values (*P*) of microarray data and fold change (FC) values of CR vs Controls. FC > 0 indicates up-regulation; FC < 0 indicates down-regulation. Threshold of significance was set at $p < 0.05$.

Gene	Forward Primer (5´to 3´)	Reverse Primer (5´to 3´)	Amplicon Size (bp)
Agrp	AGAGTTCTCAGGTCTAAGTCT	CTTGAAGAAGCGGCAGTAGCACGT	210
Il1b	AAAAATGCCTCGTGCTGTCT	GGTGTGCCGTCTTTCATCA	190
IL10	CCTGCTCTTACTGGCTGGA	GCCTGGGGCATCACTTCTAC	267
Pomc	CCTGTGAAGGTGTACCCCAATGTC	CACGTTCTTGATGATGGCGTTC	266
Rpl24	CCAAGAGGAATCAGAAACCAGA	TGGGAGCAGAGACCTTCACA	180
Rpl41	AAAGATGAGGCAGAGGTCCA	CTGTTTTGGTTGTGAGGAAGG	194
Rpl36al	AAGCACCAGCCTCACAAAGT	GTCGCCTCCCAGTTCAAAA	240

Table 4.3.3b. Nucleotide sequences of primers and amplicon size used for qRT-PCR of the selected genes to confirm the microarray analysis. *Rpl24, Rpl4* and *Rpl36al* were used as reference genes. Abbreviations: Agouti-related peptide *(AgRP),* interleukin 1 beta (*Il1b),* interleukin 10 (*Il10),* proopiomelanocortin *(Pomc),* ribosomal protein L24 *(Rpl24),* ribosomal protein L41 *(Rpl41),* ribosomal protein L36a like (*Rpl36al).*

4.3.3. Discussion

In the present study, we searched for genes whose expression levels in PBMCs of adult rats were affected by calorie restriction of their mothers during gestation and, at the same time, to assess if such alterations were totally or partially reverted by early life intervention with leptin supplementation orally taken during the suckling period. Since we were interested in predictive biomarkers in adult animals, we analyzed gene expression in adulthood before the phenotypic changes related to the metabolic syndrome were manifested. From this starting point, the whole genome transcriptome analysis in PBMCs allowed the identification of a "core group" of 401 known genes at four months of age. A follow-up of the same cohort of animals until the age of 6 months (chapter 1) revealed that CR animals, particularly when exposed to an obesogenic diet,

showed an imbalance in energy homeostasis, and displayed excess fat accumulation, insulin resistance, hypertriglyceridemia, hepatic lipid deposition and adipose tissue inflammation. These alterations were reversed, at least in part, in CR animals that were treated with oral leptin during the suckling period (chapter 1). For this reason and looking for biological plausibility, within the core group of genes we focused on genes related to feeding control and lipid metabolism, since changes in the expression levels of these genes could be tentatively considered as potential markers of predisposition to alterations in the aforementioned biological processes.

Interestingly, within the core group of genes, two of them were related to feeding control, *Agrp* (agouti-related neuropeptide), and *Pomc* (proopiomelanocortin), which is notable considering the known biological functions of the neuropeptides encoded by these genes. AgRP exhibits orexigenic effects and α-melanocyte-stimulating hormone (α-MHS), which is the cleavage product of the precursor protein POMC, inhibits food intake. Leptin has opposite effects on both pathways inhibiting the AgRP neurons and facilitating transmission in the POMC system (Pinto et al., 2004). Notably, microarray findings in PBMCs showed that the expression levels of *Agrp* were up-regulated in CR animals compared with control animals, while the expression levels of *Pomc* were down-regulated. An alteration in feeding control may be one of the mechanisms responsible for the increased susceptibility to excess weight in animals exposed to undernutrition during gestation (Bellinger et al., 2006). In support, scarcity of food in pregnant women during the Dutch hunger winter in World War II led to offspring developing obesity and associated type 2 diabetes in later life (de Rooij et al., 2006; Kahn et al., 2009; Ravelli et al., 1998). In turn, leptin supplementation during the suckling period has been previously described to have long-term effects on hypothalamic expression of *Pomc* (Pico et al., 2007), which was related to changes in the methylation pattern of the promoter (Palou et al., 2011a). This change was proposed as one of the mechanisms by which leptin intake during the lactation period programs a better resistance to obesity development in adulthood, enhancing the capacity to control food intake when animals are exposed to a high-fat diet (Palou et al., 2011a).

There were 14 genes within the core group related to lipid metabolism. Of special interest are *Pcyt2* (phosphate cytidylyltransferase 2, ethanolamine), and *Rxrβ* (retinoid X receptor beta), which were both up-regulated in CR animals compared with controls. Notably, low expression levels of *Pcyt2* and *Rxrβ* in PBMCs were proposed as potential markers of improved metabolic health since they were found to be down-regulated in the offspring of rat dams exposed to a mild calorie restriction during lactation (Konieczna et al., 2014), which are less prone to develop obesity and insulin resistance under high-fat diet feeding (Palou et al., 2010b). Thus, the opposite pattern of expression of both genes in animal models of impaired and improved metabolic health reinforces the potential interest of these genes as biomarkers.

The protein encoded by *Pcyt2* is the main regulatory enzyme in de novo biosynthesis of phosphatidylenthanolamine from ethanolamine and diacylglycerol. *Pcyt2* is known to play a pivotal role in processes which when deregulated could lead to the development of obesity, insulin resistance, liver steatosis, and dyslipidemia (Pavlovic and Bakovic,

2013). However, further studies need to be done to interrogate the potential relationship between increased expression levels of this gene and the detrimental health effects described in CR animals. Regarding *Rxrβ*, it encodes for one of the three retinoid X receptor (RXR) subtypes and belongs to the family of nuclear receptors which are involved in mediating the effects of 9-cis-retinoic acid. RXRβ heterodimerizes with peroxisome proliferator-activated receptor alpha (PPARα), and together they contribute to the induction of the acyl-CoA oxidase gene, which encodes the rate-limiting enzyme of peroxisomal ß-oxidation of fatty acids (Keller et al., 1993). RXRβ may also be involved in lipogenesis, since it mediates the induction of the lipogenic enzyme fatty acid synthase (Roder et al., 2007). The higher expression levels of this gene in PBMCs of CR animals compared with controls could be indicative of increased lipogenesis capacity in these animals, according to the positive correlation described between expression levels of this gene in PBMCs and in white adipose tissue in adult rats (Konieczna et al., 2014).

Other interesting genes related to lipid metabolism, but also with important connections with insulin homeostasis, are *Insig1* (Insulin induced gene-1) and *Npc1* (The Niemann-Pick disease, type C1). *Insig1* encodes for an endoplasmic reticulum resident protein, which inhibits the rate-limiting enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, and of SREBPs, thus preventing high-glucose-induced activation of genes for lipid biosynthesis. Therefore, it plays a critical role in the feedback control of lipid synthesis (Engelking et al., 2004). Overexpression of *Insig1* has been shown to protect β cells against glucolipotoxicity, i.e., the combined effects of high glucose and high lipid on β cell dysfunction (Engelking et al., 2004). Taking these previous findings into account, decreased expression levels of this gene in CR animals compared with controls may be in accordance with alterations in lipid metabolism and predisposition to insulin resistance under a high-fat, high-glucose diet, reinforcing its use as a potential biomarker of predisposition to alterations in lipid metabolism and insulin homeostasis.

Npc1 encodes a transmembrane protein involved in cholesterol efflux from the lysosome, but it also influences body weight and insulin homeostasis. In humans, genetic variants within NPC1 have been associated with obesity and type 2 diabetes (Al-Daghri et al., 2012; Mariman et al., 2015), and rare loss-of-function NPC1 mutations are also linked to increased risk of obesity (Liu et al., 2017). Npc1 haploinsufficient mice also display greater susceptibility to increased whole body and abdominal adiposity and hepatic steatosis, together with other abnormal metabolic features such as hyperleptinemia, dyslipidemia and insulin resistance, in the absence of hyperphagia (Jelinek et al., 2011). Alterations associated with a deficiency in NPC1 protein have been related to an impairment of insulin signaling and insulin-mediated glucose transport in adipocytes (Fletcher et al., 2014). Notably, such alterations are common with the metabolic profile of the adult offspring of gestational calorie-restricted offspring rats described by ourselves (chapter 1) and others (Desai et al., 2007), with the outputs depending on the severity of the intervention and of postnatal dietary conditions. Hence, taken together, these results provide biological bases for the

potential interest of blood cells transcript levels of *Npc1* as a biomarker of energy homeostasis and adipocyte insulin sensitivity maintenance.

Another gene of potential interest within the group of lipid-metabolism related genes is *Slc27a4* (Solute Carrier Family 27 Member 4), which has been suggested to play a role in acquired obesity (Gertow et al., 2004). This gene encodes FATP4, a member of a family of fatty acid transport proteins involved in translocation of long-chain fatty acids across the plasma membrane and intracellular fatty acid trafficking, especially transporting adipocyte lipolysis-derived fatty acids from the lipid droplet into circulation. Here, we found increased expression levels of *Slc27a4* in PBMCs of CR animals. Interestingly, expression levels of this gene in human adipose tissue were also found to be increased in obesity (independent of genetic factors), and correlated with measures of obesity and insulin resistance that are influenced by both genetic and non-genetic factors (Gertow et al., 2004).

Other genes related to lipid metabolism were also of interest in relation to cardiovascular heart, particularly *Apoo* (Apolipoprotein O), *Pla2g2a* (Secretory Phospholipase A2 Group IIA), and *Sort1* (sortilin 1), all of them upregulated in CR animals. APOO was identified as a novel apoprotein overexpressed in heart tissue in a model of obesity-related hypertension induced by a high-fat diet in dogs (Philip-Couderc et al., 2003). This protein was also found to be up-regulated in the heart of diabetic patients (Lamant et al., 2006). Further studies have demonstrated that APOO localizes in the mitochondria of cardiomyocytes and its overexpression enhances mitochondrial uncoupling and respiration, leading to mitochondrial dysfunction, lipotoxicity and cardiomyopathy (Turkieh et al., 2014). This protein has been proposed as a new signal regulator of mitochondrial function and cardiac metabolism, representing a link between impaired mitochondrial function and the onset of cardiomyopathy (Turkieh et al., 2014). Present results showing increased expression levels of *Apoo* in PBMCs of CR animals could be indicative of impaired mitochondrial function, and hence may be predictive of the altered heart function associated to obesity and diabetes, although further investigation is needed to ascertain such association in this animal model. In turn, *Pla2g2a* encodes for a member of the phospholipase A2 family. It catalyses the hydrolysis of the sn-2 ester bond of phospholipids and cell membranes, generating non-esterified free fatty acids and lyso-phospholipids, which may promote diverse pro-inflammatory processes (Six and Dennis, 2000). PLA2 type IIA is recognized for its role in chronic inflammatory diseases and generation of PGE2 and other eicosanoids after immune cell activation (Iyer, Lim et al. 2012). Remarkably, *Pla2g2a* has been described to be upregulated in the stromal vascular cell fraction of adipose tissue in diet-induced obese rats, whereas the inhibition of PLA2 type IIA protein reversed and protected against diet-induced adiposity, insulin resistance, and other metabolic dysfunctions induced by high fat diet (Iyer et al., 2012). In humans, increased plasma concentration of PLA2 type IIA has also been associated with the risk of coronary events in various studies (Hartford et al., 2006; Kugiyama et al., 1999). In line with these published studies, present finding showing a marked up-regulation of the *Pla2g2a* gene in CR animals compared to controls are in favor of the potential use of

transcript levels of this gene as a biomarker of increased susceptibility to alterations related to the metabolic syndrome, particularly cardiovascular health, but whether this may provide additional information to protein levels is not known. Sortilin 1, the protein encoded by the *Sort1* gene, is a cellular trafficking receptor that is responsible for the transport of apoB100 to the lysosome for degradation in liver. Notably, sortilin is also expressed in macrophages, where it promotes uptake of native low-density lipoprotein, foam cell formation, and atherosclerosis (Patel et al., 2015). Deficient macrophage expression of *Sort1* has been shown to be protective against atherosclerosis, at least in part by reducing LDL uptake (Patel et al., 2015), thus the finding of increased expression levels of *Sort1* in PBMCs of CR animals may be indicative of increased vascular risk, in line with previous published results.

Other genes included in the core group and related with lipid metabolism were *Acad10* (Acylcoenzyme A dehydrogenase 10), *Acot2* (Acyl-CoA thioesterase 2), *Dgkg* (Diacylglycerol kinase, gamma), *Liph* (lipase, member H), *Sar1a* (Secretion associated, Ras related GTPase 1A), and *Tm7sf2* (Transmembrane 7 superfamily member 2), but significance of changes in gene expression in PBMCs is not clear. However, it must be mentioned that some of these genes harbor genetic variations related to obesity and type 2 diabetes, such as *Dgkg* (Cheung et al., 2010), or type 2 diabetes only, such as *Acad10* (Bian et al., 2010), hence reinforcing their potential interest as candidate biomarkers.

4.3.4. Conclusions

Functional microarray analysis of PBMCs of animals which underwent prenatal and early postnatal interventions has allowed the identification of a core group of genes of interest as potential markers in adulthood of predisposition to alterations related to the metabolic syndrome and its reversion by nutritional intervention in early life. Among these genes, the focus has been on genes related to feeding control (*Agrp*, *Pomc*) and lipid metabolism with clear connections with insulin homeostasis, hepatic steatosis, and cardiovascular health (particularly *Pcyt2*, *Rxrβ*, *Insig1*, *Npc1*, *Slc27a4*, *Apoo*, *Pla2g2a*, *Sort1*), considering their plausibility according to existing biological knowledge. The interest of the biomarkers identified in the present study, if validated in humans, lies in the possibility of diagnosing the risk of obesity and metabolic-related pathologies in adulthood, before phenotypic alterations are manifested.

5. INTEGRATED DISCUSSION

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It is well established in scientific literature that maternal undernutrition has many adverse outcomes in homeostasis maintenance, leading to metabolic disorders such as hyperphagia, greater fat accumulation (Palou et al., 2010a), impaired insulin and leptin sensitivity (Palou et al., 2012), alterations in the structure and function of neuropeptides responsible of feeding behavior regulation in the hypothalamus (Garcia et al., 2010; Ikenasio-Thorpe et al., 2007) and in adipose tissue function and stomach sympathetic innervations (Garcia et al., 2011; Garcia et al., 2013). Results derived from previous studies show that leptin intervention to neonate rats throughout the suckling period is able to prevent the effects related to age and high-fat diet feeding (Pico et al., 2007; Priego et al., 2010; Sanchez et al., 2008), and reverts malprogramming effects on hypothalamic structure at early ages (Konieczna et al., 2013) and on WAT innervation and function (Konieczna et al., 2015a) associated to a mild calorie restriction during gestation.

The results shown in this doctoral thesis, included in three original manuscripts, describe that leptin supplementation throughout lactation in rat pups prevents the dysmetabolic phenotype in adulthood associated to mild calorie restriction during pregnancy and improves metabolic adaptations to an obesogenic diet. We also demonstrate that leptin supplementation during lactation reverses the decreased sympathetic drive to the stomach and normalizes the leptin/ghrelin ratio, which are altered in the offspring of calorie restricted dams during gestation. Moreover, this model was used to identify transcriptome-based biomarkers in adulthood as potential indicators of predisposition to alterations related to the metabolic syndrome and sensitive to its reversion by nutritional intervention in early life. Therefore, results obtained in this thesis bring additional evidence supporting the relevance of the intake of appropriate amounts of leptin throughout lactation to reverse postnatal sequelae induced by deficient fetal nutrition, and hence it could be worth considering it in the search of strategies designed to treat and/or prevent the programmed trend to obesity.

Our results show that mild (20%) maternal calorie restriction during the first half of gestation predisposes offspring to higher adiposity and to metabolic disturbances in adulthood. More specifically, adult offspring of calorie restricted dams during gestation (CR) displayed a higher feed efficiency, and lower energy expenditure and locomotive activity compared to control animals, suggesting an imbalance in energy homeostasis toward a positive energy balance that may explain their higher body fat accumulation. In addition to differences in energy expenditure, the increased fat accumulation observed in CR animals was suggested to be related with a decreased use of fat as a metabolic substrate, inferred from the RER values under standard diet (SD) conditions. CR animals under SD also presented diminished circulating NEFA and decreased expression levels of the lipolysis-related gene *Pnpla2* in WAT compared with control animals, suggesting a lower capacity of fat mobilization. Notably, most of these alterations were normalized in CR animals supplemented with oral leptin during the suckling period (CR-Leptin) (**Chapter 4.1**).

Energy homeostasis dysregulation occurring in adult CR animals can be largely explained by programming of insulin and leptin resistance due to undernutrition during gestation, according to previous results (Palou et al., 2010b). In fact adult CR animals displayed higher HOMA-IR index and higher plasma leptin levels than controls and CR-Leptin animals. CR animals, but not CR-Leptin ones, also displayed lower levels of hepatic AKT in accordance with an impairment of insulin sensitivity. In turn, the reduction in insulin sensitivity observed in CR animals may largely account for the dysregulation in liver lipogenesis and the impaired adaptation to a hypercaloric diet. This was evidenced by the altered hepatic expression pattern of energy metabolism-related genes regulated by insulin. Notably, the expression pattern in CR-Leptin animals was more similar to that exhibited by control animals, which is in agreement with the normalization of circulating parameters together with the lower presence of signs of hepatic steatosis when exposed to WD conditions. Moreover, gene expression analysis in WAT revealed that CR, but not CR-Leptin, animals displayed indicators of lower capacity for WAT expansion, along with decreased lipogenesis and lipolytic capacity under SD, in accordance with diminished insulin sensitivity and leptin signaling. Therefore, CR animals are less prepared for an efficient processing and handling of fuels, with an impaired capacity for lipid mobilization and clearance from circulation, and hence animals are more prone to accumulate ectopic fat in tissues other than adipose tissue. These alterations were totally or partially reverted in CR-Leptin animals (**Chapter 4.1**).

Among the mechanisms responsible for phenotypic alterations associated to undernutrition during gestation, previous studies from our group described the presence of a decreased sympathetic drive to the stomach in 25 days-old rats (Garcia et al., 2013). This alteration was suggested to be responsible for the presence of lower circulating ghrelin levels, according to the involvement of sympathetic stimulation on gastric ghrelin secretion. Here we addressed the potential reversion of this alteration by leptin treatment during lactation. Thus, we confirmed, in a different cohort of animals, the presence of a reduced sympathetic drive (i.e. lower density of $TyrOH^+$ fibers and lower TyrOH levels) in the stomach of 25 day-old CR animals. Notably, these alterations were not found in CR-Leptin animals, suggesting that the altered sympathetic innervation was reverted by leptin treatment. We also found that the alteration in sympathetic drive found at a juvenile age was less marked and not significant at the age of 6 months; however, the positive effects of leptin supplementation were still evident, since the trend to a lower density of $TyrOH^+$ fibers in CR animals compared to controls was reverted in CR-Leptin animals. Moreover, leptin treatment during the suckling period restored the diminished circulating ghrelin levels found in CR animals, although differences between groups were only significant at the age of 3 months. This study also supported that the cause of the presence of lower circulating ghrelin levels found in CR animals was the lower gastric ghrelin secretion (because of the lower sympathetic drive) and was not due to lower ghrelin production by the stomach, because no differences were found between groups concerning their mRNA expression levels, either at the age of 25 days or at 6 months (**Chapter 4.2**).

Besides individual measures of leptin and ghrelin, the leptin/ghrelin (L/G) ratio was considered of interest. In this regard, adult CR animals displayed significantly greater values of the L/G ratio than controls, in accordance to the increased tendency of these animals to develop metabolic syndrome characteristics, particularly when exposed to an obesogenic diet, as described in chapter 4.1. Notably, this ratio was completely normalised in CR-Leptin animals. These findings support the potential usefulness of the L/G ratio measurement in adulthood as a clinical non-invasive marker of obesity-related metabolic alterations, being more sensitive than fasting levels of both hormones individually; furthermore, it also seems to be able to detect the reversion of these disruptions by early life treatment (**Chapter 4.2**).

The identification of new reliable biomarkers of predisposition to chronic diseases, particularly obesity and its related alterations, could help to implement preventive strategies and decrease their prevalence (Musaad and Haynes, 2007). We performed a whole genome transcriptome analysis of PBMCs samples from the adult CR and of CR rats supplemented with leptin during the suckling period, together with their controls, to identify novel potential biomarkers in adult age indicative of a higher risk to develop alterations related to the metabolic syndrome and also sensitive to its reversion by nutritional intervention during early life. Since we were interested in predictive biomarkers in adult animals, the study was performed in adulthood, but before the phenotypic changes related to the metabolic syndrome were manifested (4 month-old animals). A group of 401 genes was found to be affected by gestational calorie restriction and reverted by leptin treatment throughout lactation. Among the genes identified, two were associated to feeding control (*Agrp* and *Pomc*), and others with lipid metabolism and with clear connections to insulin homeostasis, hepatic steatosis, and cardiovascular health according to existing biological knowledge (*Pcyt2*, *Rxrβ*, *Insig1*, *Npc1*, *Slc27a4*, *Apoo*, *Pla2g2a* and *Sort1*). These findings, if validated in humans, reveal the possibility of using transcript levels of these genes for diagnosing the risk of obesity and metabolic-related pathologies in adulthood, before phenotypic alterations are manifested, allowing the possibility of early intervention. They also bring interesting expectations for future investigations in humans (**Chapter 4.3**).

6. CONCLUSIONS

6. CONCLUSIONS

1. **Oral leptin supplementation during lactation improves energy homeostasis and metabolic control in adult rats jeopardized by undernutrition during gestation.** More specifically, supplementation with physiological doses of oral leptin throughout the suckling period was able to largely prevent the higher risk of accumulating excess fat and other disturbances of the metabolic syndrome, such as insulin resistance, hypertriglyceridemia, hepatic lipid deposition and adipose tissue inflammation, all characteristic of the offspring of mild (20%) calorie restricted dams during gestation (CR animals), particularly when exposed to obesogenic conditions in adulthood.

2. **The treatment with physiological doses of oral leptin throughout lactation to the offspring of gestational calorie-restricted dams (CR-Leptin animals) reverses the decreased sympathetic innervation in the stomach associated to maternal undernutrition.** Accordingly, leptin treatment also allowed restoring plasma ghrelin levels that were decreased in CR animals, possibly due to the impaired gastric ghrelin secretion because of the lower sympathetic innervation.

3. **CR animals displayed significantly greater values of the leptin/ghrelin (L/G) ratio than controls, in accordance to the increased tendency of these animals to develop metabolic syndrome characteristics in adulthood, particularly when exposed to an obesogenic diet**. **This ratio was completely normalized in CR-Leptin animals.** These findings are in favor of the potential usefulness of the L/G ratio measurement in adulthood as a clinical non-invasive marker of programmed susceptibility to develop obesity-related alterations; furthermore, this marker also seems to be able to detect the reversion of these disruptions by early life treatment, being more sensitive than leptin and ghrelin alone.

4. **Functional microarray analysis of PBMCs of the above-mentioned animal models, which underwent prenatal and early postnatal interventions, has allowed the identification of a core group of genes of interest as potential markers in adulthood of predisposition to alterations related to the metabolic syndrome and sensitive to its reversion by nutritional intervention with oral leptin in early life**. Among the genes identified, the focus has been on genes involved in feeding control, lipid metabolism and with clear connections to insulin homeostasis, hepatic steatosis, and cardiovascular health. The interest of these biomarkers, if validated in humans, lies in the possibility of diagnosing the risk of obesity and metabolic-related pathologies in adulthood, before phenotypic alterations are manifested.

7. REFERENCES

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8. ANNEX

8.1. ANNEX I

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

Gestational moderate calorie restriction programs offspring for later metabolic disturbances in thyroid and sympathetic signaling leading a lower BAT thermogenic capacity.

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. The aimed was 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.

8.1.1. Background

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) (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). 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 (Cannon and Nedergaard, 2004; Palou et al., 1998). 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.

8.1.2. Materials and 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, Priego 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 µ g 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 Biosciences, 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 *β-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 $P<0.05$.

8.1.3. Results

Study 1

As previously described in another group of animals(Garcia, Palou 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 8.1.1. 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.

		Males		Females		ANOVA
		Control	CR	Control	CR	
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 *	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)	

Table 8.1.1. Study 1: Circulating parameters. 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 8.1.1A 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 8.1.1C. 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 8.1.2 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.

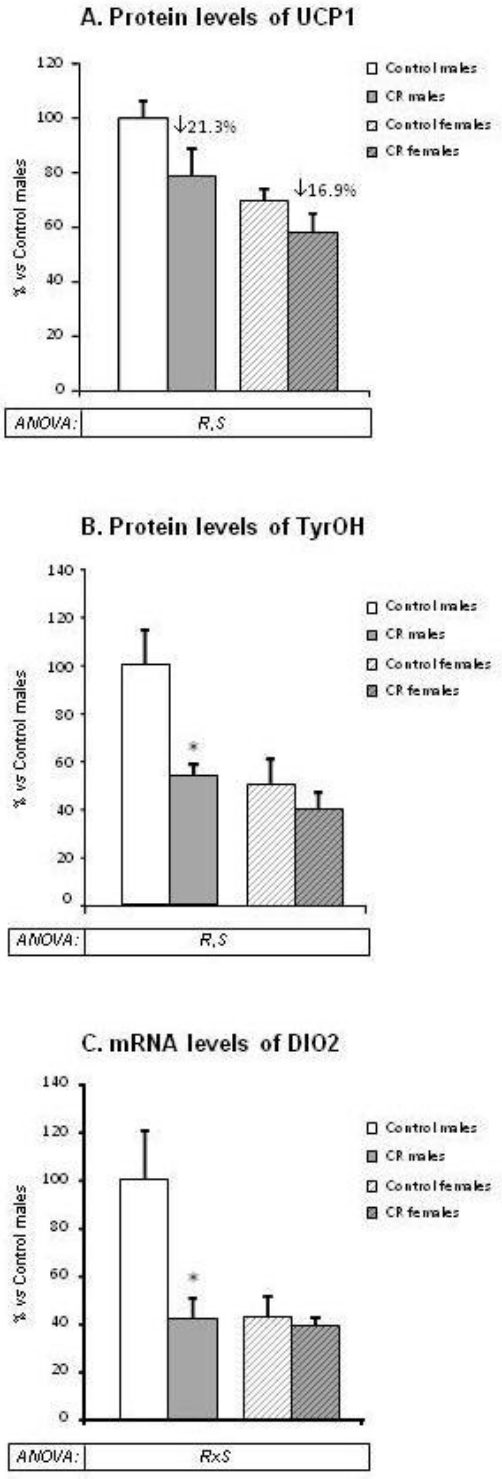

Figure 8.1.1. Specific abundance of uncoupling protein 1 (UCP1) (A) and of tyrosine hydroxylase protein (TyrOH) (B), and mRNA levels of deiodinase, iodothyronine, type II (DIO2) (C), in brown adipose tissue of 25-day-old male and female offspring from control and calorie-restricted dams during gestation (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).

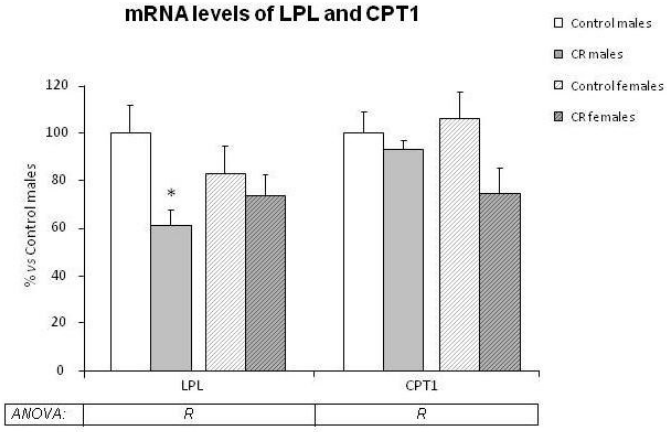

Figure 8.1.2. mRNA levels of lipoprotein lipase (LPL) and carnitine palmitoyltransferase 1 b (CPT1) in brown adipose tissue of 25-day-old male and female offspring from control and calorie-restricted dams during gestation (CR). Data are expressed as a percentage of the mean value of control male rats. 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 8.1.2 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.

	Males		**Females**		**ANOVA**
	Control (8)	**CR (10)**	**Control (8)**	**CR (10)**	
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	

Table 8.1.2. Study 2: Weight-related parameters. 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 8.1.3 shows body temperature during cold exposure in control and CR rats. In males (Figure 8.1.3A), 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 8.1.3B). 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.

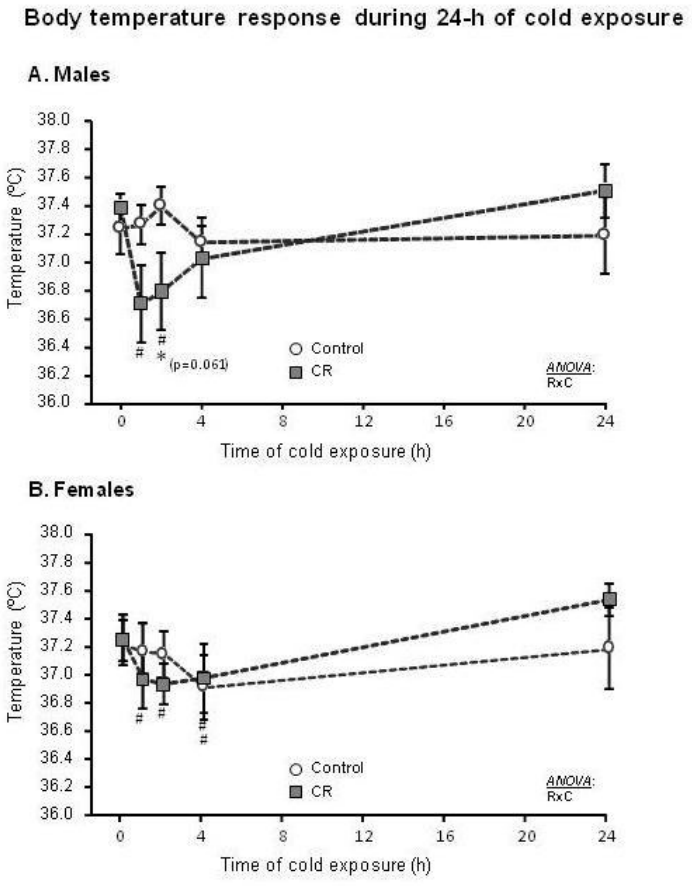

Figure 8.1.3. Body temperature of male and female offspring from control and calorie-restricted dams during gestation (CR) over 24 hours cold exposure (from day 24 to day 25 of life), measured at different time-points: before cold exposure (T0) and after 1- (T1), 2- (T2), 4- (T4) and 24-h (T24). Data 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).

Specific UCP1 protein levels in BAT of control and CR animals after 24-h cold exposure are shown in Figure 8.1.4. 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.

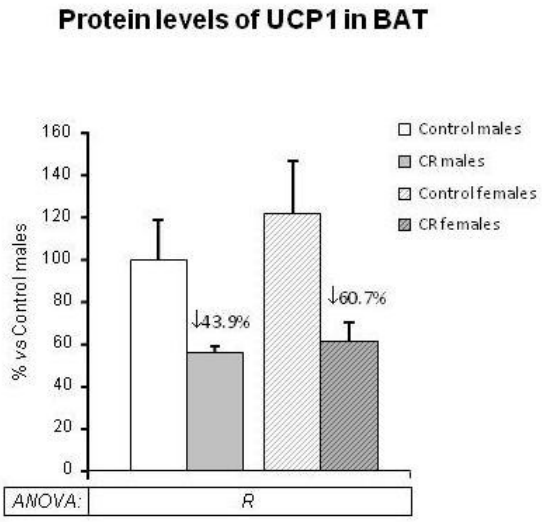

Figure 8.1.4. Specific abundance of uncoupling protein 1 (UCP1) in brown adipose tissue of 25-day-old male and female offspring from control and calorie-restricted dams during gestation (CR) after 24 hours of cold exposure. Data are expressed as a percentage of the mean value of control rats. Data are 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).

8.1.4. 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 (Nedergaard and Cannon, 2010; Tam et al., 2012). 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, Priego 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,

Priego 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 (Nedergaard et al., 1995; Palou et al., 1998). 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 innervation (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 impair 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 innervation (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., 2001; Ribeiro et al., 2000). 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 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).

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.

8.1.5. Conclusions

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 occurs 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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8.2. ANNEX II

Detailed list of genes related to blood, cell communication, cell turnover, cytoskeleton, immune system, metabolism (amino acids/polyamines), metabolism (carbohydrates), metabolism (central), metabolism (inositol), metabolism (nucleic acid/nucleotides), metabolism (proteins), metabolism (redox), metabolism (vitamins/minerals), miscellanea, nervous system, neuronal signaling, signaling, transcription/translation machinery, transport and vascular homeostasis included in the 401 "core genes" differentially expressed in CR vs control animals and simultaneously not different between control and CR-Leptin animals of the study described in **Section 4.3**.

Biological process	Gene symbol	Gene name	Sequence ID	Microarray					
				CR vs C		CRL vs CR		CRL vs C	
				p-value	Fold change	*p*-value	Fold change	*p*-value	Fold change
Blood	Fiz1	FLT3-interacting zinc finger 1	NM_001106223	0.035	1.08	0.121	0.94	0.676	1.01
	Pbx2	Pre-B-cell leukemia homeobox 2	NM_001002828	0.014	1.10	0.143	0.95	0.356	1.04
Cell comunication	Adam10	ADAM metallopeptidase domain 10	XR_356412	0.014	1.22	0.398	0.94	0.101	1.15
	Cpne3	Copine III	XM_006237932	0.031	1.14	0.740	0.98	0.105	1.12
	Fbn1	Fibrillin 1	NM_031825	0.023	0.70	0.193	1.21	0.288	0.84
	Itga6	Integrin, alpha 6	BE110753	0.026	1.31	0.370	0.86	0.505	1.13
	Itgae	Integrin, alpha E	NM_031768	0.005	1.19	0.200	0.94	0.072	1.12
	Ocln	Occludin	NM_031329	0.047	1.13	0.264	0.92	0.557	1.04
	Prnp	Prion protein	NM_012631	0.005	1.28	0.309	0.92	0.114	1.17
	Tpbg	Trophoblast glycoprotein	NM_031807	0.037	1.16	0.984	1.00	0.090	1.16
	Zfyve21	Zinc finger, FYVE domain containing 21	XM_006240639	0.031	1.25	0.897	0.98	0.076	1.23
Cell turnover	Arid2	AT rich interactive domain 2 (Arid-rfx like)	XM_345867	0.012	1.12	0.505	0.97	0.073	1.09
	Bcl2l1	Bcl2-like 1	NM_001033670	0.042	1.19	0.997	1.00	0.151	1.19
	Bod1	Biorientation of chromosomes in cell division 1	NM_001013854	0.042	1.08	0.171	0.96	0.403	1.03
	Bop1	Block of proliferation 1	NM_001024250	0.035	1.13	0.277	0.96	0.194	1.08

Casp4	Caspase 4, apoptosis-related cysteine peptidase	NM_053736	0.013	0.88	0.443	1.04	0.120	0.92
Casp8ap2	Caspase 8 associated protein 2	NM_001107921	0.048	1.06	0.811	0.99	0.179	1.05
Cdc45	Cell division cycle 45	NM_001105866	0.033	1.10	0.941	1.00	0.069	1.09
Cdk5rap1	CDK5 regulatory subunit associated protein 1	NM_145721	0.022	1.17	0.279	0.94	0.183	1.09
Cdkn1a	Cyclin-dependent kinase inhibitor 1A	NM_080782	0.027	0.74	0.339	1.16	0.318	0.85
Cenpa	Centromere protein A	NM_001106711	0.046	0.85	0.035	1.11	0.466	0.95
Cep295	Centrosomal protein 295	NM_001271048	0.017	0.95	0.690	0.99	0.052	0.94
Crlf3	Cytokine receptor-like factor 3	AF072835	0.019	1.13	0.707	0.98	0.085	1.11
Dmtf1	Cyclin D binding myb-like transcription factor 1	NM_053693	0.024	1.07	0.246	0.97	0.353	1.04
Dtx1	Deltex 1, E3 ubiquitin ligase	XM_001076559	0.010	1.29	0.202	0.90	0.147	1.15
E2f1	E2F transcription factor 1	NM_001100778	0.025	1.14	0.285	0.96	0.095	1.09
Eepd1	Endonuclease/exonuclease/phosphatase family domain containing 1	NM_001014088	0.027	1.40	0.291	0.84	0.291	1.18
Egln3	Egl-9 family hypoxia-inducible factor 3	NM_019371	0.033	1.23	0.348	0.86	0.658	1.07
Ets2	V-ets avian erythroblastosis virus E26 oncogene homolog 2	NM_001107107	0.019	0.82	0.238	1.11	0.304	0.91
Fancc	Fanconi anemia, complementation group C	NM_012557	0.033	1.17	0.550	0.97	0.074	1.13
Fas	Fas cell surface death receptor	NM_139194	0.001	0.77	0.144	1.14	0.116	0.88
G0s2	G0/G1switch 2	CA505509	0.035	0.80	0.116	1.24	0.978	1.00
Haus8	HAUS augmin-like complex, subunit 8	NM_001024971	0.004	1.13	0.960	1.00	0.068	1.13
Junb	Jun B proto-oncogene	NM_021836	0.019	0.80	0.670	1.05	0.181	0.84
Katnal1	Katanin p60 subunit A-like 1	NM_001006956	0.048	1.82	0.137	0.64	0.359	1.17
Klf10	Kruppel-like factor 10	NM_031135	0.013	0.82	0.446	1.07	0.191	0.88
Kras	Kirsten rat sarcoma viral oncogene	XM_008763354	0.036	0.90	0.260	1.05	0.265	0.94
Lamtor3	Late endosomal/lysosomal adaptor, MAPK and MTOR activator 3	NM_001008375	0.014	0.88	0.085	1.07	0.213	0.95
Mina	Myc induced nuclear antigen	NM_153309	0.011	1.12	0.138	0.93	0.312	1.04
Mnt	MAX network transcriptional repressor	NM_001105807	0.033	0.90	0.031	1.09	0.743	0.99
Mtdh	Metadherin	NM_133398	0.042	0.90	0.795	1.02	0.151	0.91

Myc	Myelocytomatosis oncogene	NM_012603	0.042	1.35	0.135	0.82	0.520	1.10
Ndrg2	NDRG family member 2	NM_133583	0.023	1.18	0.271	0.90	0.464	1.06
Ndrg3	NDRG family member 3	NM_001013923	0.048	1.14	0.237	0.94	0.262	1.08
Nedd1	Neural precursor cell expressed, developmentally down-regulated 1	NM_001106779	0.020	0.91	0.324	1.03	0.146	0.94
Nucks1	Nuclear casein kinase and cyclin-dependent kinase substrate 1	NM_022799	0.042	0.87	0.586	1.04	0.223	0.90
Pds5b	PDS5 cohesin associated factor B	NM_001102383	0.025	0.92	0.748	1.01	0.138	0.94
Poc5	POC5 centriolar protein	NM_001025647	0.048	0.89	0.413	1.05	0.248	0.94
Ppp1r15a	Protein phosphatase 1, regulatory subunit 15A	NM_133546	0.005	0.84	0.757	1.02	0.051	0.86
Pwp1	PWP1 homolog (S. cerevisiae)	NM_001191877	0.039	1.13	0.717	0.98	0.056	1.11
Rab11fip4	RAB11 family interacting protein 4 (class II)	XM_006246903	0.047	1.09	0.378	0.95	0.469	1.04
Rab19	RAB19, member RAS oncogene family	NM_001024326	0.046	1.14	0.526	0.92	0.745	1.05
Rbm24	RNA binding motif protein 24	NM_001191100	0.025	1.15	0.297	0.94	0.135	1.09
Rbm45	RNA binding motif protein 45	NM_153306	0.042	1.08	0.678	0.98	0.069	1.06
Rmdn3	Regulator of microtubule dynamics 3	NM_001014046	0.009	0.90	0.027	1.10	0.912	1.00
Rmnd5b	Required for meiotic nuclear division 5 homolog B (S. cerevisiae)	NM_001017473	0.015	1.23	0.504	0.96	0.066	1.18
Rnf130	Ring finger protein 130	NM_001037658	0.015	0.92	0.699	1.01	0.051	0.93
Rpa2	Replication protein A2	NM_021582	0.027	1.11	0.895	0.99	0.146	1.10
Rprd1a	Regulation of nuclear pre-mRNA domain containing 1A	NM_001305179	0.003	1.27	0.109	0.90	0.090	1.14
Sdccag3	Serologically defined colon cancer antigen 3	NM_001013135	0.045	1.14	0.592	0.98	0.100	1.12
Ski	SKI proto-oncogene	XM_008764420	0.040	1.16	0.901	0.99	0.078	1.15
Spin1	Spindlin 1	NM_001024796	0.027	0.90	0.417	1.03	0.082	0.92
Spint2	Serine peptidase inhibitor, Kunitz type, 2	NM_199087	0.027	1.11	0.880	0.99	0.094	1.10
Tsc22d1	TSC22 domain family, member 1	NM_001109912	0.042	1.58	0.225	0.66	0.925	1.03
Tsnax	Translin-associated factor X	NM_022262	0.026	1.19	0.125	0.90	0.400	1.07
Xiap	X-linked inhibitor of apoptosis	XM_008773529	0.027	1.10	0.430	0.96	0.372	1.05
Zranb3	Zinc finger, RAN-binding domain containing 3	XM_006249702	0.007	1.13	0.184	0.95	0.144	1.06

Cytoskeleton	Cald1	Caldesmon 1	NM_013146	0.042	1.56	0.178	0.71	0.730	1.10
	Cdc42ep4	CDC42 effector protein (Rho GTPase binding) 4	NM_001107063	0.045	0.76	0.802	1.02	0.052	0.77
	Clip1	CAP-GLY domain containing linker protein 1	NM_031745	0.036	0.84	0.298	1.08	0.137	0.91
	Cotl1	Coactosin-like F-actin binding protein 1	NM_001108452	0.046	1.13	0.960	1.00	0.054	1.13
	Ezr	Ezrin	NM_019357	0.038	0.88	0.563	1.03	0.127	0.91
	Flii	Flightless I homolog (Drosophila)	NM_001008279	0.041	1.12	0.849	0.99	0.090	1.11
	Gsn	Gelsolin	NM_001004080	0.024	1.45	0.436	0.86	0.293	1.25
	Iqgap2	IQ motif containing GTPase activating protein 2	XM_002729002	0.030	1.12	0.806	0.99	0.101	1.11
	Padi6	Peptidyl arginine deiminase, type VI	NM_001191766	0.044	0.87	0.267	1.06	0.200	0.92
	Specc1l	Sperm antigen with calponin homology and coiled-coil domains 1-like	XM_006256339	0.048	0.93	0.329	1.04	0.574	0.97
	Sptbn1	Spectrin, beta, non-erythrocytic 1	NM_001013130	0.010	1.21	0.334	0.93	0.165	1.13
	Tpm1	Tropomyosin 1, alpha	NM_001034069	0.023	1.17	0.369	0.94	0.278	1.09
	Tubb5	Tubulin, beta 5 class I	NM_173102	0.011	1.17	0.532	0.95	0.208	1.11
	Tube1	Tubulin, epsilon 1	NM_001108536	0.030	1.10	0.668	0.98	0.213	1.08
	Tubgcp3	Tubulin, gamma complex associated protein 3	XM_006253440	0.039	0.92	0.993	1.00	0.079	0.92
Immune system	Aoah	Acyloxyacyl hydrolase (neutrophil)	NM_001191940	0.029	0.83	0.117	1.16	0.590	0.96
	Atrn	Attractin	NM_031351	0.030	0.89	0.923	1.01	0.092	0.90
	B2m	Beta-2 microglobulin	NM_012512	0.047	1.27	0.618	0.93	0.269	1.18
	Ccl6	Chemokine (C-C motif) ligand 6	BC079460	0.049	0.75	0.275	1.13	0.263	0.85
	Ccr10	Chemokine (C-C motif) receptor 10	NM_001108836	0.026	1.19	0.408	0.94	0.156	1.12
	Cd7	Cd7 molecule	NM_001107074	0.047	1.21	0.741	0.96	0.171	1.16
	Ctsw	Cathepsin W	NM_001024242	0.018	1.29	0.881	0.99	0.060	1.27
	Cyba	Cytochrome b-245, alpha polypeptide	NM_024160	0.033	0.84	0.051	1.12	0.396	0.93
	Fcgr1a	Fc fragment of IgG, high affinity Ia, receptor (CD64)	AF416291	0.020	0.82	0.239	1.12	0.355	0.92
	Ifih1	Interferon induced with helicase C domain 1	NM_001109199	0.020	0.90	0.524	1.03	0.133	0.93
	Il10	Interleukin 10	NM_012854	0.027	0.80	0.418	1.07	0.119	0.85
	Il1b	Interleukin 1 beta	NM_031512	0.002	0.54	0.433	1.22	0.115	0.66

	Il36b	Interleukin 36, beta	NM_001108570	0.030	0.83	0.590	1.05	0.108	0.87
	Lilrb3	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	NM_031713	0.045	0.83	0.368	1.10	0.348	0.91
	Lilrb4	Leukocyte immunoglobulin-like receptor, subfamily B, member 4	NM_001013894	0.012	0.79	0.325	1.10	0.174	0.87
	Mrc1	Mannose receptor, C type 1	NM_001106123	0.042	1.29	0.503	0.95	0.080	1.23
	Plcl2	Phospholipase C-like 2	NM_001106880	0.030	0.90	0.178	1.06	0.401	0.96
	Pou2f2	POU class 2 homeobox 2	NM_001271204	0.021	0.82	0.277	1.09	0.257	0.90
	Ripk2	Receptor-interacting serine-threonine kinase 2	NM_001191865	0.025	0.90	0.328	1.04	0.070	0.94
	Samsn1	SAM domain, SH3 domain and nuclear localization signals, 1	NM_130821	0.003	0.78	0.249	1.12	0.146	0.87
	Sema4a	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4A	NM_001012078	0.034	1.12	0.138	0.93	0.521	1.04
	Sftpd	Surfactant protein D	NM_012878	0.016	1.53	0.385	0.78	0.571	1.19
	Tlr2	Toll-like receptor 2	NM_198769	0.001	0.70	0.254	1.14	0.078	0.80
	Tlr7	Toll-like receptor 7	NM_001097582	0.019	0.82	0.556	1.06	0.168	0.87
	Tnfrsf18	Tumor necrosis factor receptor superfamily, member 18	NM_001024349	0.005	1.17	0.650	0.97	0.076	1.14
	Tnfrsf26	Tumor necrosis factor receptor superfamily, member 26	NM_001108511	0.013	0.86	0.129	1.07	0.184	0.93
	Trim26	Tripartite motif-containing 26	NM_001011665	0.011	0.87	0.407	1.04	0.094	0.91
	Wdr34	WD repeat domain 34	NM_001005542	0.043	1.12	0.653	0.98	0.106	1.09
Metabolism (amino acids/polyamines)	Ass1	Argininosuccinate synthase 1	NM_013157	0.037	0.79	0.497	1.08	0.160	0.86
	Azin1	Antizyme inhibitor 1	NM_022585	0.031	0.91	0.948	1.00	0.062	0.90
Metabolism (carbohydrates)	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	NM_017008	0.013	1.22	0.684	0.95	0.271	1.16
	Hexa	Hexosaminidase A	NM_001004443	0.032	1.08	0.928	1.00	0.069	1.09
	Pfkfb4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	NM_019333	0.050	0.90	0.464	1.05	0.482	0.95
	Pgm2l1	Phosphoglucomutase 2-like 1	NM_001109454	0.034	1.11	0.097	0.93	0.568	1.03
	Pkm	Pyruvate kinase, muscle	NM_053297	0.023	1.16	0.835	1.02	0.066	1.18

	Pxylp1	Phosphoxylose phosphatase 1	NM_001007710	0.025	1.12	0.544	0.97	0.137	1.09
	Slc16a3	Solute carrier family 16 (monocarboxylate transporter), member 3	NM_030834	0.015	0.71	0.179	1.19	0.259	0.84
	Slc2a8	Slc2a8|solute carrier family 2, (facilitated glucose transporter) member 8	NM_053494	0.017	1.13	0.076	0.90	0.836	1.01
	Slc2a9	Slc2a9|solute carrier family 2 (facilitated glucose transporter), member 9	NM_001191551	0.018	0.84	0.146	1.07	0.104	0.89
	Uxs1	Uxs1|UDP-glucuronate decarboxylase 1	NM_139336	0.038	1.14	0.908	0.99	0.050	1.13
Metabolism (central)	Ndufaf5	NADH dehydrogenase (ubiquinone) complex I, assembly factor 5	NM_001126371	0.031	1.07	0.912	1.00	0.077	1.06
	Timmdc1	Translocase of inner mitochondrial membrane domain containing 1	NM_001007676	0.013	1.13	0.631	0.98	0.051	1.11
Metabolism (inositol)	Isyna1	Inositol-3-phosphate synthase 1	NM_001013880	0.042	1.13	0.910	0.99	0.139	1.12
	Pi4ka	Phosphatidylinositol 4-kinase, catalytic, alpha	NM_022301	0.025	0.90	0.756	1.01	0.052	0.91
	Pi4kb	Phosphatidylinositol 4-kinase, catalytic, beta	NM_031083	0.029	1.12	0.289	0.97	0.110	1.09
	Pip4k2b	Phosphatidylinositol-5-phosphate 4-kinase, type II, beta	XM_008768131	0.038	1.11	0.748	0.98	0.140	1.09
	Pitpnb	Phosphatidylinositol transfer protein, beta	NM_053742	0.049	0.91	0.561	1.03	0.121	0.94
	Tmem55a	Transmembrane protein 55A	NM_001024900	0.012	1.28	0.522	0.93	0.092	1.19
Metabolism (nucleic acid/nucleotides)	Atic	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	NM_031014	0.000	1.17	0.063	0.92	0.086	1.08
	Dnase2b	Deoxyribonuclease II beta	NM_021664	0.016	0.60	0.241	1.24	0.204	0.75
	Nme1	NME/NM23 nucleoside diphosphate kinase 1	NM_138548	0.036	1.09	0.919	1.00	0.117	1.08
	Nt5c3a	5'-nucleotidase, cytosolic IIIA	NM_001107862	0.037	1.43	0.384	0.85	0.313	1.22
	Prpsap1	Phosphoribosyl pyrophosphate synthetase-associated protein 1	NM_022545	0.032	1.16	0.558	0.96	0.226	1.11
	Rrm2	Ribonucleotide reductase M2	NM_001025740	0.034	1.13	0.880	1.01	0.055	1.14
Metabolism (proteins)	Atg3	Autophagy related 3	NM_134394	0.023	0.92	0.250	1.04	0.121	0.95
	B3galt5	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5	NM_001105887	0.024	0.77	0.437	1.09	0.086	0.84
	Cct8	Chaperonin containing Tcp1, subunit 8 (theta)	NM_001105897	0.044	0.90	0.841	0.99	0.070	0.90

Cpq	Carboxypeptidase Q	NM_031640	0.022	1.52	0.396	0.84	0.249	1.29
Dcun1d4	DCN1, defective in cullin neddylation 1, domain containing 4	NM_001108359	0.003	1.16	0.545	0.96	0.094	1.11
Dzip3	DAZ interacting zinc finger protein 3	XM_006221123	0.026	1.15	0.056	0.91	0.450	1.05
Eefsec	Eukaryotic elongation factor, selenocysteine-tRNA-specific	NM_001109249	0.026	0.89	0.503	1.03	0.129	0.92
Fam76a	Family with sequence similarity 76, member A	NM_001108686	0.040	0.91	0.947	1.00	0.067	0.90
Fbxo31	F-box protein 31	NM_001044259	0.006	1.26	0.047	0.88	0.184	1.11
Gpaa1	Glycosylphosphatidylinositol anchor attachment 1	NM_001004240	0.025	1.13	0.583	0.98	0.053	1.11
Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member 1	NM_001004082	0.047	1.15	0.614	0.97	0.068	1.12
Isca2	Iron-sulfur cluster assembly 2	NM_001109278	0.039	0.86	0.072	1.08	0.264	0.93
Mkrn1	Makorin ring finger protein 1	XM_006236301	0.036	1.29	0.428	0.89	0.362	1.15
Napsa	Napsin A aspartic peptidase	NM_031670	0.029	0.81	0.389	1.07	0.129	0.87
Ogt	O-linked N-acetylglucosamine (GlcNAc) transferase	NM_017107	0.018	1.17	0.314	0.94	0.144	1.10
Psma6	Proteasome (prosome, macropain) subunit, alpha type 6	NM_017283	0.003	0.84	0.085	1.07	0.053	0.90
Psmg2	Proteasome (prosome, macropain) assembly chaperone 2	NM_001106138	0.020	1.08	0.794	0.99	0.078	1.07
Secisbp2	SECIS binding protein 2	NM_024002	0.046	1.07	0.769	0.99	0.301	1.05
Stt3b	STT3B, subunit of the oligosaccharyltransferase complex (catalytic)	NM_001170539	0.038	0.86	0.695	1.02	0.054	0.88
Trnau1ap	TRNA selenocysteine 1 associated protein 1	NM_023027	0.029	0.90	0.106	1.07	0.400	0.96
Tspan17	Tetraspanin 17	NM_001013138	0.014	1.28	0.742	0.96	0.137	1.22
Uba5	Ubiquitin-like modifier activating enzyme 5	NM_001009669	0.032	0.90	0.913	1.01	0.056	0.91
Ubap1l	Ubiquitin associated protein 1-like	XM_008776739	0.009	0.87	0.380	1.05	0.108	0.92
Ube2e2	Ubiquitin-conjugating enzyme E2E 2	NM_001108371	0.040	1.23	0.427	0.92	0.273	1.14
Ube4b	Ubiquitination factor E4B	NM_001271198	0.001	1.16	0.171	0.93	0.086	1.08
Ubqln2	Ubiquilin 2	NM_001108251	0.036	0.86	0.536	1.03	0.098	0.89
Ubxn1	UBX domain protein 1	NM_001034829	0.029	0.92	0.070	1.05	0.289	0.96
Ufl1	Ufm1-specific ligase 1	NM_001126279	0.024	0.90	0.696	1.02	0.067	0.92

	Usp10	Ubiquitin specific peptidase 10	NM_001034146	0.018	1.11	0.324	0.96	0.126	1.06
	Usp16	Ubiquitin specific peptidase 16	NM_001100501	0.012	1.12	0.633	0.98	0.065	1.10
	Usp19	Ubiquitin specific peptidase 19	NM_001001516	0.016	1.14	0.516	0.97	0.052	1.10
	Usp44	Ubiquitin specific peptidase 44	NM_001108083	0.014	1.15	0.400	0.96	0.083	1.10
	Usp46	Ubiquitin specific peptidase 46	NM_001191596	0.006	1.29	0.330	0.90	0.174	1.16
	Wwp1	WW domain containing E3 ubiquitin protein ligase 1	NM_001024757	0.011	0.90	0.757	0.98	0.063	0.89
Metabolism (redox)	Cyb5b	Cytochrome b5 type B (outer mitochondrial membrane)	Y12517	0.022	1.22	0.282	0.94	0.088	1.14
	Me2	Malic enzyme 2, NAD(+)-dependent, mitochondrial	NM_001107376	0.048	0.95	0.103	1.06	0.728	1.01
	Mgst1	Microsomal glutathione S-transferase 1	NM_134349	0.042	0.86	0.932	0.99	0.067	0.86
	Nfe2l2	Nuclear factor, erythroid 2-like 2	NM_031789	0.010	0.83	0.425	1.06	0.066	0.88
	Oplah	5-oxoprolinase (ATP-hydrolysing)	NM_053904	0.018	1.22	0.426	0.92	0.182	1.12
	Sqrdl	Sulfide quinone reductase-like (yeast)	NM_001047913	0.016	0.83	0.336	1.09	0.259	0.90
	Tmx1	Thioredoxin-related transmembrane protein 1	NM_001024800	0.010	1.11	0.509	0.98	0.053	1.08
	Zadh2	Zinc binding alcohol dehydrogenase, domain containing 2	NM_001106129	0.030	1.20	0.458	0.96	0.122	1.15
Metabolism (vitamins/minerals)	Fam20c	Family with sequence similarity 20, member C	NM_001012238	0.026	0.76	0.565	1.05	0.063	0.80
	Folr2	Folate receptor 2 (fetal)	NM_001106283	0.028	0.93	0.398	1.02	0.193	0.96
	Hlcs	Holocarboxylase synthetase (biotin-(proprionyl-CoA-carboxylase (ATP-hydrolysing)) ligase)	XM_006221086	0.018	1.14	0.382	0.96	0.138	1.09
	Mmachc	Methylmalonic aciduria (cobalamin deficiency) cblC type, with homocystinuria	NM_001107962	0.025	1.13	0.562	0.97	0.051	1.11
	Pdxk	Pyridoxal (pyridoxine, vitamin B6) kinase	NM_031769	0.022	0.90	0.910	1.01	0.058	0.90
Miscelanea	Bfsp2	Beaded filament structural protein 2, phakinin	NM_001277434	0.022	1.30	0.887	0.98	0.127	1.28
	Bhlhe40	Basic helix-loop-helix family, member e40	NM_053328	0.006	0.77	0.190	1.14	0.189	0.87
	Car7	Carbonic anhydrase 7	NM_001106165	0.040	1.41	0.877	1.03	0.070	1.45
	Ccdc47	Coiled-coil domain containing 47	NM_001013974	0.021	1.17	0.321	0.96	0.088	1.12
	Ggt1	Gamma-glutamyltransferase 1	NM_053840	0.020	1.12	0.802	0.99	0.062	1.10
	Hapln2	Hyaluronan and proteoglycan link protein 2	NM_022285	0.038	1.12	0.985	1.00	0.053	1.12

	Kdm6b	Lysine (K)-specific demethylase 6B	NM_001108829	0.014	0.83	0.145	1.10	0.272	0.92
	Sparc	Secreted protein, acidic, cysteine-rich (osteonectin)	NM_012656	0.037	1.38	0.193	0.86	0.209	1.19
	Tbx2	T-box 2	NM_001107033	0.033	0.86	0.338	1.06	0.083	0.91
Nervous system	Bex1	Brain expressed, X-linked 1	NM_001037365	0.024	1.14	0.439	0.93	0.513	1.06
	Bhlhb9	Basic helix-loop-helix domain containing, class B, 9	NM_207611	0.046	1.11	0.578	0.98	0.134	1.09
	Cntf	Ciliary neurotrophic factor	NM_013166	0.034	1.13	0.507	0.98	0.079	1.10
	Dbn1	Drebrin 1	NM_031024	0.025	1.21	0.534	0.97	0.052	1.18
	Ngdn	Neuroguidin, EIF4E binding protein	NM_001107263	0.041	0.90	0.872	1.01	0.096	0.91
	Ralgps2	Ral GEF with PH domain and SH3 binding motif 2	NM_001100680	0.023	0.90	0.741	1.02	0.221	0.92
	Sarm1	Sterile alpha and TIR motif containing 1	NM_001105817	0.046	1.09	0.100	0.94	0.678	1.02
	Sema6d	Sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	NM_001107768	0.024	1.41	0.311	0.80	0.606	1.12
Neuronal signaling	Abhd12	Abhydrolase domain containing 12	NM_001024314	0.016	0.88	0.761	1.02	0.081	0.89
	Chn1	Chimerin 1	NM_032083	0.024	1.22	0.519	0.93	0.297	1.13
	Grin1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	NM_017010	0.023	0.93	0.292	1.04	0.260	0.96
	Grm2	Glutamate receptor, metabotropic 2	NM_001105711	0.050	1.11	0.755	0.99	0.066	1.09
	Kctd12	Potassium channel tetramerization domain containing 12	XM_006222049	0.033	1.32	0.607	0.94	0.141	1.24
	Oat	Ornithine aminotransferase	NM_022521	0.037	0.91	0.767	1.02	0.258	0.93
	Ppfia4	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 4	NM_080409	0.024	1.26	0.396	0.91	0.221	1.15
	Slc6a4	Solute carrier family 6 (neurotransmitter transporter), member 4	NM_013034	0.040	1.46	0.242	0.76	0.646	1.12
	Snapin	SNAP-associated protein	NM_001025648	0.022	0.91	0.681	1.02	0.126	0.93
	Zmynd19	Zinc finger, MYND-type containing 19	NM_198770	0.011	1.18	0.323	0.94	0.075	1.11
Signaling	Adcy7	Adenylate cyclase 7	NM_053396	0.022	1.27	0.729	1.06	0.055	1.34
	Adgra2	Adhesion G protein-coupled receptor A2	XM_003751617	0.031	1.27	0.310	0.88	0.430	1.11

Akap7	A kinase (PRKA) anchor protein 7	NM_001001801	0.024	0.93	0.179	1.07	0.942	1.00	
Ankmy2	Ankyrin repeat and MYND domain containing 2	NM_001108019	0.028	1.10	0.053	0.92	0.678	1.02	
Anxa3	Annexin A3	NM_012823	0.028	0.60	0.805	1.08	0.105	0.65	
Ar	Androgen receptor	NM_012502	0.013	1.11	0.111	0.94	0.273	1.04	
Areg	Amphiregulin	NM_017123	0.034	0.79	0.595	1.06	0.074	0.84	
Arhgap28	Rho GTPase activating protein 28	XM_006245626	0.031	1.63	0.182	0.65	0.866	1.06	
Bgn	Biglycan	NM_017087	0.046	1.57	0.240	0.72	0.681	1.13	
Bmp2	Bone morphogenetic protein 2	NM_017178	0.019	0.82	0.482	1.06	0.101	0.86	
Bmpr2	Bone morphogenetic protein receptor, type II (serine/threonine kinase)	NM_080407	0.018	0.86	0.441	1.04	0.108	0.89	
Brap	BRCA1 associated protein	NM_001271194	0.026	0.89	0.455	1.04	0.165	0.92	
Calm2	Calmodulin 2	NM_017326	0.026	0.87	0.188	1.06	0.140	0.92	
Deptor	DEP domain containing MTOR-interacting protein	XM_001066889	0.035	0.87	0.255	1.08	0.318	0.94	
Dok2	Docking protein 2	NM_001106048	0.005	1.15	0.093	0.93	0.123	1.08	
Dok4	Docking protein 4	XM_008772317	0.015	1.65	0.463	0.85	0.101	1.40	
Dusp16	Dual specificity phosphatase 16	XM_008763374	0.005	0.91	0.814	1.01	0.051	0.92	
Gab2	GRB2-associated binding protein 2	XM_008759806	0.017	0.85	0.565	1.04	0.119	0.89	
Gpr107	G protein-coupled receptor 107	NM_001107828	0.005	0.91	0.367	1.03	0.112	0.94	
Gprasp1	G protein-coupled receptor associated sorting protein 1	NM_134386	0.025	1.19	0.126	0.92	0.232	1.10	
Gpsm1	G-protein signaling modulator 1	NM_144745	0.043	1.10	0.914	1.01	0.073	1.10	
Igfbp4	Insulin-like growth factor binding protein 4	NM_001004274	0.048	1.22	0.175	0.93	0.189	1.13	
Lancl2	LanC lantibiotic synthetase component C-like 2 (bacterial)	XM_006236581	0.049	1.12	0.500	0.96	0.259	1.08	
Ldlrad4	Low density lipoprotein receptor class A domain containing 4	XM_008772172	0.037	0.90	0.276	1.05	0.300	0.95	
Lyve1	Lymphatic vessel endothelial hyaluronan receptor 1	NM_001106286	0.008	1.54	0.828	0.96	0.064	1.47	
Map3k5	Mitogen-activated protein kinase kinase kinase 5	NM_001277694	0.038	0.91	0.390	1.06	0.668	0.97	
Map3k9	Mitogen-activated protein kinase kinase kinase 9	NM_001100872	0.028	0.88	0.673	1.03	0.148	0.91	
Mapk3	Mitogen activated protein kinase 3	NM_017347	0.047	0.90	0.476	1.05	0.419	0.95	

	Mark3	MAP/microtubule affinity-regulating kinase 3	NM_130749	0.009	0.91	0.815	1.01	0.066	0.91
	Mcf2l	MCF.2 cell line derived transforming sequence-like	XM_006253419	0.037	1.17	0.853	0.98	0.132	1.15
	Nf1	Neurofibromin 1	NM_012609	0.004	0.86	0.027	1.12	0.460	0.96
	Nkiras1	NFKB inhibitor interacting Ras-like 1	NM_001107252	0.040	1.10	0.609	0.98	0.144	1.08
	Pi4k2a	Phosphatidylinositol 4-kinase type 2 alpha	NM_053735	0.039	0.88	0.692	1.02	0.063	0.89
	Prkcb	Protein kinase C, beta	NM_001172305	0.045	0.88	0.529	1.02	0.074	0.90
	Ptk2	Protein tyrosine kinase 2	NM_013081	0.017	1.28	0.692	0.95	0.127	1.22
	Ptpre	Protein tyrosine phosphatase, receptor type, E	NM_053767	0.001	0.82	0.320	1.07	0.062	0.88
	Rasa4	RAS p21 protein activator 4	XM_002724809	0.031	1.24	0.660	0.94	0.284	1.17
	Rasgrp3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	NM_001108009	0.037	1.54	0.135	0.65	0.995	1.00
	Rassf2	Ras association (RalGDS/AF-6) domain family member 2	NM_001037096	0.042	0.90	0.999	1.00	0.082	0.90
	Rbpj	Recombination signal binding protein for immunoglobulin kappa J region	NM_001106631	0.028	0.85	0.357	1.07	0.210	0.91
	Rhobtb1	Rho-related BTB domain containing 1	NM_001107622	0.015	1.15	0.472	0.96	0.152	1.10
	Sesn3	Sestrin 3	NM_001108125	0.028	1.18	0.412	0.95	0.092	1.12
	Smad7	SMAD family member 7	NM_030858	0.039	0.63	0.550	1.11	0.090	0.70
	Stat5a	Signal transducer and activator of transcription 5A	NM_017064	0.026	1.11	0.984	1.00	0.084	1.11
	Tti1	TELO2 interacting protein 1	NM_001134619	0.046	0.89	0.778	0.99	0.054	0.88
	Ywhah	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	NM_013052	0.016	1.39	0.336	0.85	0.329	1.18
Transcription/translation machinery	Ankrd13c	Ankyrin repeat domain 13C	NM_001191570	0.046	0.89	0.874	1.01	0.079	0.89
	Apbb1	Amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)	NM_080478	0.011	1.12	0.999	1.00	0.074	1.12
	Atf1	Activating transcription factor 1	NM_001100895	0.044	1.08	0.388	0.97	0.190	1.05
	Ctdp1	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosphatase, subunit 1	NM_001106131	0.017	0.79	0.113	1.12	0.171	0.89
	Cux1	Cut-like homeobox 1	XM_008760271	0.038	1.08	0.939	1.00	0.064	1.09
	Dcp2	Decapping mRNA 2	NM_001170469	0.024	1.17	0.564	0.97	0.080	1.13

Ddx39b	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39B	NM_133300	0.036	1.13	0.515	0.97	0.096	1.10	
Dhx38	DEAH (Asp-Glu-Ala-His) box polypeptide 38	NM_001106185	0.014	0.92	0.760	0.99	0.061	0.91	
Dnajb6	DnaJ (Hsp40) homolog, subfamily B, member 6	NM_001013209	0.026	1.11	0.237	0.95	0.298	1.06	
Dnajc1	DnaJ (Hsp40) homolog, subfamily C, member 1	XM_002728514	0.045	1.11	0.987	1.00	0.068	1.11	
Drosha	Drosha, ribonuclease type III	XM_006232049	0.043	1.22	0.716	0.97	0.063	1.18	
Eif4a1	Eukaryotic translation initiation factor 4A1	NM_199372	0.019	1.08	0.864	0.99	0.095	1.08	
Eif4h	Eukaryotic translation initiation factor 4H	NM_001006957	0.003	0.87	0.171	1.05	0.071	0.92	
Emg1	EMG1 N1-specific pseudouridine methyltransferase	NM_001107888	0.045	1.13	0.711	0.98	0.140	1.10	
Fau	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed	NM_001012739	0.045	0.89	0.292	1.04	0.156	0.92	
Fhl2	Four and a half LIM domains 2	NM_031677	0.046	1.17	0.052	0.87	0.885	1.01	
Foxk2	Forkhead box K2	XM_006247890	0.014	0.93	0.212	1.04	0.457	0.97	
G3bp1	GTPase activating protein (SH3 domain) binding protein 1	NM_133565	0.037	1.10	0.935	1.00	0.109	1.09	
G3bp2	GTPase activating protein (SH3 domain) binding protein 2	XM_006250736	0.022	1.14	0.352	0.93	0.427	1.06	
Gfm2	G elongation factor, mitochondrial 2	NM_001100665	0.016	1.09	0.206	0.95	0.303	1.03	
Gnl3l	Guanine nucleotide binding protein-like 3 (nucleolar)-like	NM_001081958	0.035	0.90	0.361	1.05	0.257	0.94	
Gpatch1	G patch domain containing 1	NM_001106246	0.014	0.88	0.463	1.04	0.065	0.92	
Hdac10	Histone deacetylase 10	NM_001035000	0.041	1.13	0.932	1.01	0.090	1.14	
Hes1	Hes family bHLH transcription factor 1	NM_024360	0.021	0.75	0.479	1.08	0.127	0.81	
Hnrnpa1	Heterogeneous nuclear ribonucleoprotein A1	NM_017248	0.023	0.73	0.213	1.14	0.192	0.84	
Ints10	Integrator complex subunit 10	XM_008771092	0.022	1.13	0.272	0.95	0.155	1.08	
Ints12	Integrator complex subunit 12	NM_001007640	0.034	0.91	0.593	1.02	0.070	0.93	
Ints7	Integrator complex subunit 7	NM_001191675	0.018	1.13	0.198	0.95	0.182	1.07	
Isy1	ISY1 splicing factor homolog (S. cerevisiae)	NM_001014188	0.021	0.91	0.684	1.02	0.165	0.93	
Klf7	Kruppel-like factor 7 (ubiquitous)	NM_001108800	0.038	0.86	0.673	1.03	0.115	0.89	
Lsm14a	LSM14A, SCD6 homolog A (S. cerevisiae)	NM_001127552	0.013	1.10	0.586	0.98	0.061	1.08	
Luc7l2	LUC7-like 2 (S. cerevisiae)	NM_001107853	0.045	1.10	0.702	0.99	0.082	1.09	

Med19	Mediator complex subunit 19	NM_001107741	0.044	1.12	0.404	0.96	0.120	1.08
Mettl10	Methyltransferase like 10	NM_001108504	0.039	0.90	0.709	1.02	0.089	0.91
Mettl15	Methyltransferase like 15	XM_008762084	0.033	1.10	0.147	0.93	0.461	1.03
Mex3c	Mex-3 RNA binding family member C	NM_001107377	0.003	1.06	0.206	0.94	0.978	1.00
Mrpl1	Mitochondrial ribosomal protein L1	NM_001105997	0.038	0.84	0.292	1.11	0.465	0.93
Mrps21	Mitochondrial ribosomal protein S21	NM_001126094	0.050	1.09	0.923	1.00	0.057	1.09
Mtfmt	Mitochondrial methionyl-tRNA formyltransferase	NM_001009697	0.031	0.91	0.893	0.99	0.053	0.91
Orc6	Origin recognition complex, subunit 6	NM_001033690	0.038	0.86	0.778	1.01	0.066	0.88
Phf5a	PHD finger protein 5A	NM_138888	0.030	0.92	0.852	1.01	0.088	0.92
Plagl2	Pleiomorphic adenoma gene-like 2	NM_001106528	0.041	0.87	0.358	1.05	0.206	0.92
Pop4	Processing of precursor 4, ribonuclease P/MRP subunit (S. cerevisiae)	NM_001009642	0.033	0.89	0.459	1.03	0.144	0.92
Pou6f1	POU class 6 homeobox 1	NM_001105746	0.029	1.12	0.877	1.01	0.073	1.13
Pprc1	Peroxisome proliferator-activated receptor gamma, coactivator-related 1	NM_001106363	0.036	1.11	0.847	0.99	0.077	1.10
Prmt2	Protein arginine methyltransferase 2	NM_001025144	0.002	1.18	0.107	0.92	0.150	1.08
Prpf8	Pre-mRNA processing factor 8	NM_001191590	0.013	0.88	0.374	1.04	0.096	0.92
Pus7	Pseudouridylate synthase 7	XM_008762634	0.004	1.15	0.361	0.96	0.061	1.10
Rnasek	Ribonuclease, RNase K	NM_001137561	0.022	1.19	0.498	0.94	0.282	1.12
Rpl15	Ribosomal protein L15	NM_139114	0.032	1.11	0.405	0.96	0.224	1.07
Rps7	Ribosomal protein S7	X56846	0.023	0.86	0.518	1.04	0.062	0.90
Rrp1b	Ribosomal RNA processing 1B	XM_228076	0.031	1.11	0.654	0.98	0.079	1.09
Safb	Scaffold attachment factor B	NM_022394	0.049	1.15	0.452	0.96	0.119	1.11
Snrpa1	Small nuclear ribonucleoprotein polypeptide A'	NM_001271323	0.016	0.88	0.584	1.03	0.093	0.91
Snrpd1	Small nuclear ribonucleoprotein D1	NM_001106163	0.038	0.90	0.751	0.98	0.050	0.88
Snrpn	Small nuclear ribonucleoprotein polypeptide N	NM_031117	0.039	0.87	0.305	1.07	0.230	0.93
Srsf1	Serine/arginine-rich splicing factor 1	NM_001109552	0.022	1.09	0.878	1.01	0.050	1.10
St13	Suppression of tumorigenicity 13	NM_031122	0.030	1.18	0.766	0.98	0.054	1.16
Stau2	Staufen double-stranded RNA binding protein 2	NM_001007149	0.047	1.34	0.329	0.83	0.546	1.12

	Sympk	Symplekin	NM_001100830	0.022	1.11	0.948	1.00	0.093	1.12
	Syncrip	Synaptotagmin binding, cytoplasmic RNA interacting protein	XM_006243515	0.039	1.11	0.087	0.93	0.530	1.03
	Taf11	TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor	NM_001008350	0.041	0.92	0.964	1.00	0.108	0.93
	Taf1c	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, C	NM_001014155	0.028	1.11	0.913	1.01	0.065	1.11
	Taf8	TAF8 RNA polymerase II, TATA box binding protein (TBP)-associated factor	NM_001108197	0.011	1.09	0.991	1.00	0.052	1.09
	Trim28	Tripartite motif-containing 28	NM_053916	0.000	1.13	0.123	0.95	0.053	1.07
	Trps1	Trichorhinophalangeal syndrome I	NM_001134837	0.017	0.88	0.495	1.04	0.122	0.92
	Tsen34	tRNA splicing endonuclease 34 homolog (S. cerevisiae)	NM_001006968	0.022	0.84	0.098	1.10	0.279	0.92
	Utp20	UTP20, small subunit (SSU) processome component, homolog (yeast)	NM_001191779	0.034	1.12	0.687	0.98	0.088	1.09
	Wwtr1	WW domain containing transcription regulator 1	NM_001024869	0.047	1.16	0.773	0.98	0.079	1.14
	Yaf2	YY1 associated factor 2	NM_001134871	0.016	1.12	0.194	0.94	0.290	1.05
	Zbed4	Zinc finger, BED-type containing 4	NM_001134800	0.045	1.20	0.945	1.01	0.119	1.21
	Zfp280d	Zinc finger protein 280D	NM_001108165	0.023	0.86	0.257	1.07	0.199	0.91
	Zfp593	Zinc finger protein 593	NM_001106689	0.020	0.89	0.876	1.01	0.063	0.89
	Zfp655	Zinc finger protein 655	NM_001008362	0.032	0.87	0.414	1.04	0.131	0.90
	Zfp868	Zinc finger protein 868	NM_001009538	0.033	0.88	0.859	1.01	0.052	0.89
Transport	Abca3	ATP-binding cassette, subfamily A (ABC1), member 3	XM_006220590	0.038	0.90	0.495	1.02	0.105	0.92
	Agfg1	ArfGAP with FG repeats 1	NM_001135596	0.043	1.08	0.700	0.99	0.144	1.06
	Ap3m2	Adaptor-related protein complex 3, mu 2 subunit	L07074	0.043	1.08	0.423	0.97	0.349	1.04
	Apol11a	Apolipoprotein L 11a	XM_006226144	0.037	0.78	0.150	1.23	0.740	0.96
	Atl3	Atlastin GTPase 3	NM_001044241	0.008	1.10	0.914	1.01	0.160	1.11
	Atp2a2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	NM_001110139	0.046	0.93	0.981	1.00	0.083	0.93
	Atp6v1d	ATPase, H+ transporting, lysosomal V1 subunit D	NM_199386	0.036	0.90	0.618	1.02	0.151	0.92

Atp7a	ATPase, Cu++ transporting, alpha polypeptide	XM_006256995	0.023	0.88	0.096	1.08	0.368	0.95	
Bet1l	Bet1 golgi vesicular membrane trafficking protein-like	NM_019368	0.020	0.89	0.647	1.02	0.093	0.91	
Clta	Clathrin, light chain A	NM_031974	0.045	0.92	0.044	1.07	0.603	0.98	
Cnst	Consortin, connexin sorting protein	XM_573525	0.027	1.37	0.267	0.87	0.257	1.19	
Ehd3	EH-domain containing 3	XM_006239774	0.026	1.23	0.726	0.97	0.073	1.19	
Esyt1	Extended synaptotagmin-like protein 1	NM_017249	0.030	1.14	0.954	1.00	0.070	1.14	
Fcho1	FCH domain only 1	NM_001106069	0.021	1.78	0.451	0.86	0.096	1.54	
Fxn	Frataxin	NM_001191952	0.045	0.90	0.538	1.03	0.142	0.93	
Hbe1	Hemoglobin, epsilon 1	NM_001008890	0.024	1.50	0.047	0.75	0.513	1.12	
Hiatl1	Hippocampus abundant transcript-like 1	NM_001107334	0.010	0.87	0.421	1.04	0.052	0.91	
Lrrc8b	Leucine rich repeat containing 8 family, member B	XM_006250557	0.015	1.12	0.511	0.97	0.075	1.09	
Napg	N-ethylmaleimide-sensitive factor attachment protein, gamma	NM_001107384	0.011	0.90	0.804	1.01	0.088	0.91	
Rab11a	RAB11a, member RAS oncogene family	NM_031152	0.043	1.16	0.735	0.97	0.133	1.12	
Rab1a	RAB1A, member RAS oncogene family	NM_031090	0.050	0.93	0.991	1.00	0.086	0.93	
Rab3il1	RAB3A interacting protein (rabin3)-like 1	NM_134411	0.037	0.80	0.718	1.03	0.053	0.83	
Rab43	RAB43, member RAS oncogene family	NM_001024331	0.005	0.86	0.101	1.08	0.209	0.93	
Rab9a	RAB9A, member RAS oncogene family	NM_053458	0.011	0.89	0.124	1.05	0.105	0.93	
Rangap1	RAN GTPase activating protein 1	NM_001244855	0.010	1.12	0.255	0.95	0.108	1.06	
Rtn3	Reticulon 3	NM_080909	0.002	1.14	0.379	0.95	0.148	1.08	
Scfd2	Sec1 family domain containing 2	NM_001017499	0.006	1.17	0.358	0.94	0.113	1.11	
Slc12a4	Solute carrier family 12 (potassium/chloride transporter), member 4	NM_019229	0.018	1.18	0.741	0.98	0.054	1.15	
Slc12a6	Solute carrier family 12, member 6	NM_001109630	0.041	0.92	0.888	0.99	0.087	0.92	
Slc19a1	Solute carrier family 19 (folate transporter), member 1	NM_017299	0.021	1.15	0.645	0.98	0.055	1.13	
Slc20a1	Solute carrier family 20 (phosphate transporter), member 1	NM_031148	0.025	0.90	0.697	1.01	0.060	0.91	
Slc22a23	Solute carrier family 22, member 23	NM_022624	0.039	1.23	0.099	0.84	0.756	1.03	

	Slc25a36	Solute carrier family 25 (pyrimidine nucleotide carrier), member 36	XM_576451	0.024	0.86	0.991	1.00	0.089	0.86
	Slc38a6	Solute carrier family 38, member 6	NM_001013099	0.013	0.85	0.630	1.04	0.149	0.89
	Slc40a1	Solute carrier family 40 (iron-regulated transporter), member 1	NM_133315	0.047	1.29	0.363	0.85	0.645	1.09
	Slc44a1	Solute carrier family 44 (choline transporter), member 1	NM_001033852	0.047	1.37	0.358	0.87	0.250	1.19
	Slc4a7	Solute carrier family 4, sodium bicarbonate cotransporter, member 7	NM_058211	0.036	0.86	0.622	1.02	0.059	0.88
	Stx12	Syntaxin 12	NM_022939	0.030	1.08	0.824	0.99	0.176	1.07
	Tbc1d14	TBC1 domain family, member 14	NM_001012152	0.035	0.88	0.721	1.02	0.084	0.90
	Tbc1d17	TBC1 domain family, member 17	NM_001106258	0.034	0.87	0.758	1.02	0.066	0.89
	Tnpo1	Transportin 1	NM_001100692	0.027	1.10	0.359	0.97	0.080	1.07
	Trappc5	Trafficking protein particle complex 5	NM_001108850	0.037	0.89	0.654	1.01	0.067	0.90
	Tspo	Translocator protein	NM_012515	0.029	0.88	0.626	1.02	0.055	0.89
	Vamp4	Vesicle-associated membrane protein 4	NM_001108856	0.043	0.90	0.798	1.01	0.084	0.91
	Vps26a	Vacuolar protein sorting 26 homolog A (S. pombe)	NM_001007740	0.004	0.90	0.251	1.04	0.073	0.93
	Vps45	Vacuolar protein sorting 45 homolog (S. cerevisiae)	NM_172072	0.008	1.17	0.199	0.93	0.154	1.08
Vascular homeostasis	Ctgf	Connective tissue growth factor	NM_022266	0.035	0.80	0.344	1.07	0.133	0.86
	Nr3c2	Nuclear receptor subfamily 3, group C, member 2	NM_013131	0.049	0.79	0.324	1.09	0.210	0.86
	P2ry12	Purinergic receptor P2Y, G-protein coupled, 12	XM_006232408	0.047	1.59	0.182	0.64	0.939	1.03
	Plaur	Plasminogen activator, urokinase receptor	NM_134352	0.015	0.74	0.046	1.40	0.873	1.03
	Ren	Renin	NM_012642	0.045	0.92	0.046	1.09	0.887	1.01
	Tfpi	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	NM_017200	0.030	1.24	0.199	0.91	0.137	1.13
	Vegfa	Vascular endothelial growth factor A	NM_031836	0.004	0.71	0.222	1.18	0.195	0.83
	Vegfc	Vascular endothelial growth factor C	NM_053653	0.040	1.12	0.509	1.06	0.055	1.19