



**Universitat de les
Illes Balears**

**DOCTORAL THESIS
2014**

**EFFECTS OF MODERATE MATERNAL ENERGY
RESTRICTION ON THE OFFSPRING METABOLIC
HEALTH, IN TERMS OF OBESITY AND RELATED
DISEASES, AND IDENTIFICATION OF
DETERMINANT FACTORS AND EARLY
BIOMARKERS**

Juana María Torrens García



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**Doctoral Programme of Nutrigenomics and
Personalized Nutrition**

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DETERMINANT FACTORS AND EARLY
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WE DECLARE:

That the thesis entitled *Effects of moderate maternal energy restriction on the offspring metabolic health, in terms of obesity and related diseases, and identification of determinant factors and early biomarkers*, presented by Juana María Torrens García 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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A mis padres

Agradecimientos

Quiero expresar mi agradecimiento a todas las personas que han contribuido, de una forma u otra, en la realización de esta tesis.

Recuerdo perfectamente el día en el que me preguntaron si quería ser alumna colaboradora del laboratorio. Yo estaba en segundo de carrera y la verdad es que por aquel entonces no había pensado en nada de doctorados ni en investigación, simplemente me hacía muchas ganas conocer cómo se trabaja en un laboratorio, así que lo probé. Empecé un verano ayudando básicamente a Ona en el estabulario con las “ratitas” y como tanto el trabajo como la gente del laboratorio me engancharon, verano tras verano volví a repetir hasta que, al acabar la carrera, decidí empezar el doctorado y continuar con lo que me gustaba, la investigación. Y aquí estoy ahora, escribiendo unas líneas para agradecer a todas las personas que han hecho posible que haya conseguido llegar hasta el final de esta etapa. La redacción nunca se me ha dado bien y mucho menos reflexionar sobre mis sentimientos, —siempre he dicho que soy de números y no de letras—, pero haré lo posible para que todo el agradecimiento que siento quede reflejado en estas líneas.

Mi especial agradecimiento a mis dos directores de tesis: el Prof. Andreu Palou y la Prof. Catalina Picó por su contribución en mi formación investigadora. Al Prof. Andreu Palou, quiero agradecerle la oportunidad que me brindó para formar parte y para realizar el doctorado en su grupo de investigación, el Laboratorio de Biología Molecular, Nutrición y Biotecnología (LBNB) de la Universidad de las Islas Baleares. También quiero agradecerle la supervisión y los consejos aportados a mi tesis, así como también la oportunidad que me ofreció para realizar mis dos estancias predoctorales en la Universidad de Warwick, en el Reino Unido. A la Prof. Catalina Picó quiero agradecerle muy especialmente, toda la confianza, paciencia y comprensión que ha tenido conmigo en todos los momentos (porque sé que soy muy cabezota, y cuando me encierro en una cosa, soy muy difícil de sacar de ahí). Quiero agradecerle toda la ayuda que me ha dado durante todos estos años, y especialmente durante estos últimos meses, en los que la redacción de la tesis ha sido un camino duro y complicado.

A los demás profesores del grupo: Dra. Paula Oliver, Prof. Francisca Serra, Dra. Ana María Rodríguez, Dr. Joan Ribot y Dra. Lluïsa Bonet, quiero agradecerles sus consejos, su ayuda y la proximidad de su trato.

Muchísimas gracias Dra. Mariona Palou y Dra. Joana Sánchez, por vuestra amistad, por escucharme, por comprenderme y por vuestras enseñanzas; sin vosotras no estaría en estos momentos a punto de terminar el doctorado. Me guiasteis en mis primeros pasos en el laboratorio: a trabajar con los animales (santa paciencia tuvisteis conmigo que siempre me desmayaba cuando teníamos que sacar sangre!!), a manejar las máquinas del laboratorio, a rellenar papeles administrativos, a escribir resultados.... Sinceramente, las dos habéis estado desde el principio siempre a mi lado y me habéis ayudado tanto a nivel profesional como personal en todos los obstáculos que han ido apareciendo a lo largo de estos años.

A la Dra. Teresa Priego, al Dr. Pep Mercader y a la Dra. Nuria Granados, gracias por vuestro apoyo, por vuestros ánimos y consejos.

Thank you to the Prof. Paul Thornalley and Dr. Naila Rabanni for giving me the opportunity to work with them, for all their advices and for treat me as another one of the group. Thanks to all the members of the Protein Damage group: Fozia, Dr. Amy, Dr. Ming, Dr. Makoto, Alla, Amal, Dr. Attia Dra. Zhera, and especially to Dr. Jinit for his patience understanding and advices (also thanks to all “Costa time”).

Quiero agradecer especialmente a Enzo por hacerme sonreír siempre que he estado triste y por hacerme reír cuando sólo quería llorar. Gracias por enseñarme morfología e histología y por ayudarme siempre que lo he necesitado.

A todos mis compañeros de laboratorio, quiero daros las gracias: Rubén y Xisco, gracias por convertir el despacho en el Polo Norte!!!, voy a añorar no discutir con vosotros por el aire acondicionado; Estefanía, simplemente gracias por todos tus detallitos; Nora, gracias por escucharme y darme consejos; Raúl, gracias por hacerme reír y saber escuchar; Petar, gracias por ser cómo eres; Madhu, gracias por cuidarnos tan bien con tus postres; Heriberto; gracias por deleitarnos con la comida mejicana; Nara, gracias por tu alegría y vitalidad; Alice, gracias por escucharme cuando lo he necesitado.

Especialmente quiero agradecer a la Dra. Jadwiga Konieczna por ayudarme siempre y por aguantarme tantas horas en el estabulario y en laboratorio.

A los nuevos becarios Cati Dora, Marga Cifre, Andrea, Alba y Sebastián, gracias, mucha suerte y muchos ánimos.

A los demás compañeros del laboratorio, Bea; al personal del estabulario, Teresa de Francisco y Miquel, gracias.

A los antiguos compañeros del laboratorio, Dr. Toni Caimari, Dr. Jaume Amengual, Dra. María Servera, Dra. Ana Paula García, Dra. Hana Musinovic y especialmente a la Dra. Pilar Parra, gracias por acogerme tan bien cuando llegué al grupo.

Quiero agradecer muy especialmente a las *Pingüinas*: Bárbara, Marina, Ona y Joana. Gracias por vuestros ánimos pero sobre todo por haberme aguantado (sé que estos últimos meses he sido *la niña del exorcista* y “mordía” por todo, pero a pesar de ello habéis seguido ahí), gracias por escucharme, por vuestro apoyo, por vuestros consejos, por reír conmigo, por llorar conmigo...por todo, gracias!!.

Quiero agradecer la ayuda de todos los alumnos colaboradores, en especial a Cristina Cabrer, Cati Joy, y Bel Mora. Gracias Cati Joy por ser mi mejor amiga (aunque hayamos estado semanas sin saber la una de la otra), gracias por aguantarme cuando he estado agobiada y por reñirme por mis tonterías. Gracias Bel, por tu amistad, por tu cariño y por tu gran ayuda, porque estos últimos meses no lo hubiese podido conseguir sin ti.

A mi prima Natalia, muchas gracias por escucharme siempre, por animarme, apoyarme y sobre todo por hacerme reír cuando he estado triste.

Finalmente, y no por ello menos importante, quiero darles las gracias a mis padres, Miguel y Antonia. Lo sois todo para mí, y todo lo que pueda decir de vosotros se quedaría corto. Os quiero mucho y si he llegado hasta aquí has sido gracias a vosotros. Gracias por vuestro esfuerzo y sacrificio incondicional para que yo pudiese estudiar. Gracias por todas las facilidades que me habéis dado. Gracias por ayudarme a estudiar y por estar a mi lado en todo momento. Gracias por aguantar mis enfados, mis lloros y mi estrés durante toda la vida y especialmente durante estos últimos meses. Os quiero agradecer todo lo que me habéis enseñado desde pequeña y que me han formado como la persona que ahora soy, solo espero no defraudaros nunca. Ahora, lo que más deseo es compartir con vosotros todo lo que he conseguido gracias a vuestra ayuda, apoyo, confianza, esfuerzo, comprensión y dedicación. *Gracias mami!!.* *Gracias papi!!!* *Esto es por y para vosotros!!!.* *Os quiero!!!.*

Guty, esta recta final no hubiese sido posible tampoco sin ti. Gracias por tu comprensión y por tu enorme paciencia estos últimos meses. Gracias por escuchar mis preocupaciones, por aguantar mis lloros y mis tonterías. Gracias por tus consejos, por tus abrazos, tus mimos, por tu ayuda, por todo. Te ha tocado conocerme en una época de mucho estrés para mí, pero a pesar de ello, sigues aguantándome. Simplemente gracias por ser cómo eres, por confiar en mí y por quererme tal y como soy. *Te quiero.*

Index

Abbreviations	VII
Abstract/Resumen/Resum	IX
List of original articles	XV
I. Introduction	1
1. Obesity.....	3
2. Energy balance regulation	4
2.1 Main tissues involved in maintenance of energy homeostasis	5
2.1.1. Hypothalamus.....	5
2.1.2. White adipose tissue.....	7
2.1.3. Liver	8
2.1.4. Skeletal muscle.....	10
2.2 Main hormones involved in maintenance of energy homeostasis	11
2.2.1. Leptin	11
<i>Leptin during the perinatal period</i>	12
<i>Leptin resistance</i>	14
2.2.2. Insulin.....	15
<i>Insulin resistance</i>	16
2.3 Calorie restriction	20
3. Developmental origins of obesity: importance of early life nutrition	21
3.1. Programming effects of maternal calorie restriction during gestation	22
3.2. Programming effects of maternal calorie restriction during lactation	24
<i>Breast milk</i>	25
4. Biomarkers of metabolic health	26
4.1. Gene expression analysis in PBMCs	27
4.2. Quantitative screening of protein damage biomarkers.	28
II. Aim and experimental design.....	33
III. Materials and methods	43
1. Experimental animals	45
2. PBMC isolation	45
3. RNA isolation and quantification.....	46
3.1. Tripure reagent method for RNA isolation	47

3.2. E.Z.N.A total RNA kit I for RNA isolation	47
3.3. RNA quantification and assessment of its integrity	48
4. Gene expression analysis by RT-qPCR	49
4.1. Reverse transcription	49
4.2. Real Time quantitative Polymerase Chain Reaction (RT-qPCR).....	49
5. Determination of circulating parameters	50
5.1. Insulin.....	50
5.2. Glucose	51
5.3. Homeostatic model assessment for insulin resistance (HOMA-IR).....	51
5.4. Leptin.....	51
5.5. Triglycerides (TGs)	52
5.6. Non-esterified fatty acids (NEFAs).....	53
5.7. β -hydroxybutirate (BHB)	53
5.8. Adiponectin	53
5.9. Tumor Necrosis Factor alpha (TNF α).....	54
6. Measurement of hepatic glycogen content	54
7. Total hepatic lipid content measurement	55
8. Measurement of hepatic triglyceride content	56
9. Histological analysis	57
10. Confocal microscopy for Green Fluorescent Protein detection in liver samples	58
11. Isolation of mitochondria from liver	58
12. Western blot analysis	59
12.1. Sample preparation.....	59
12.2. Electrophoresis	60
12.3. Electroblothing	60
12.4. Labelling and detection	61
12.5. Scanning and quantification	62
12.6. Loading control	62
13. Indirect gas calorimetry and locomotive activity measurements	62
14. Blood pressure measurement	63
15. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)	63
15.1. Sample preparation.....	64
15.2. Ultrahigh Performance Liquid Chromatography (UPLC).....	64

15.3. MS/MS detection.....	64
15.4. Calculation.....	64
16. Statistical analysis	65
IV. Results and discussion	67
1. Impaired insulin and leptin sensitivity in the offspring of moderate caloric-restricted dams during gestation is early programmed	69
2. Enhancing hepatic fatty acid oxidation as a strategy for reversing metabolic disorders programmed by maternal undernutrition during gestation.....	95
3. Moderate caloric restriction in lactating rats protects offspring against obesity and insulin resistance in later life.....	121
4. Moderate caloric restriction in lactating rats programs their offspring for a better response to HF diet feeding in a sex-dependent manner.....	141
5. Early biomarkers identified in a rat model of a healthier phenotype based on early postnatal dietary intervention may predict the response to an obesogenic environment in adulthood	163
6. Identification of early transcriptome-based biomarkers related to lipid metabolism in peripheral blood mononuclear cells of rats nutritionally programmed for improved metabolic health.....	185
7. Moderate maternal calorie restriction in lactating rats affects markers of protein damage by glycation and oxidation in breast milk	209
V. Recapitulation.....	227
VI. Conclusions/Conclusiones	235
VII. Bibliography	241

Abbreviations

3DG-H: 3-deoxyglucosone
3-NT: 3-Nitrotyrosine
AAV: adeno-associated virus
ACC: acetyl-CoA carboxylase
ACOX: acyl-CoA oxidase
AdipoR1: adiponectin receptor 1
AdipoR2: adiponectin receptor 2
AGEs: advanced glycation endproducts
AgRP: Agouti-related peptide
AKT: protein kinase B (PKB)
AMPK: AMP-activated protein kinase
 α -MSH: alpha-melanocyte-stimulating hormone
ARC: arcuate nucleus
ATGL: adipose tissue triglyceride lipase
BMI: body mass index
cAMP: cyclic adenosine monophosphate
CART: cocaine and amphetamine-regulated transcript
CEL: N ϵ -carboxyethyl-lysine
CKM: creatine kinase
CMA: N ω -carboxymethylarginine
CML: N ϵ -carboxymethyl-lysine
CNS: central nervous system
CPT1: carnitine palmitoyltransferase 1
CPT1A: carnitine palmitoyltransferase 1 liver isoform
CPT1AM: mutant form of carnitine palmitoyltransferase 1 liver isoform
CRH: corticotropin releasing hormone
DEX: dexamethasone
DMN: dorsomedial nucleus
FABP: fatty acid binding protein
FAO: fatty acid oxidation
FASC: fatty acid synthase complex
FASN: fatty acid synthase
FATP: fatty acid binding protein
FL: Fructosyl-lysine
FTO: fat mass and obesity associated
GCK: glucokinase
G-H1: N δ -(5-hydro-4-imidazolone-2-yl)ornithine
GLUT4: glucose transporter 4
GPAT: glycerol-3-phosphate acyltransferase
GSA: glutamic semialdehyde
HDL: high-density lipoprotein
HF: high fat
HSL: hormone-sensitive lipase
IDL: intermediate density lipoproteins
IL-6: interleukin-6
InsR: insulin receptor

IRS: insulin receptor substrate
IRX3: iroquois homeobox 3 gene
iWAT: inguinal white adipose tissue
JAK2: janus-activated tyrosine kinase 2
LCAS: long chain acyl-CoA synthetase
LC-MS/MS: liquid chromatography with tandem mass spectrometry detection
LDL: low-density lipoprotein
LH: lateral hypothalamus
LPL: lipoprotein lipase
MC3R: melanocortin receptor subtype 3
MC4R: melanocortin receptor subtype 4
MCH: melanin-concentrating hormone
MetSO: methionine sulfoxide
MG-H1: N δ -(5-hydro-5-methyl-4-imidazol-2-yl)ornithine
MOLD: bis(lysyl) crosslink derived from methylglyoxal
NEFAs: non-esterified fatty acids
NFK: N-formylkynurenine
NPY: neuropeptide Y
ObRb: long form of leptin receptor
PBMCs: peripheral blood mononuclear cells
PCK1: phosphoenolpyruvate carboxykinase
PDK1: 3-phosphoinositide-dependent protein kinase 1
PGC1 α : peroxisome-proliferator-activated receptor gamma co-activator 1 alpha
PI3K: phosphatidylinositol-3-kinase
PIP2: phosphatidylinositol-4,5-bisphosphate
PIP3: phosphatidylinositol-3,4,5-triphosphate
PKA: protein kinase A
PKL: liver pyruvate kinase
POMC: pro-opiomelanocortin
PPAR α : peroxisome-proliferator-activated receptor alpha
PPAR γ 2: peroxisome-proliferator-activated receptor gamma-2
PVN: paraventricular nucleus
rWAT: retroperitoneal white adipose tissue
SOCS3: suppressor of cytokine signaling
SREBP1c: sterol-regulatory-element-binding protein
STAT3: signal transducer and activator of transcription 3
TCA: tricarboxylic acid
TG: triglyceride
TNF α : tumor necrosis factor alpha
TRH: thyrotropin releasing hormone
VLDL: very low density lipoprotein
VMN: ventromedial nucleus
WAT: white adipose tissue
WHO: World Health Organization



Effects of moderate maternal energy restriction on the offspring metabolic health, in terms of obesity and related diseases, and identification of determinant factors and early biomarkers

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Abstract

A growing body of evidence, from epidemiological studies in humans and animal models, indicate that maternal health and nutritional status during gestation and lactation can program the propensity to develop obesity in their offspring. Huge efforts are now being directed toward understanding the molecular mechanisms underlying this developmental programming. Identification of these mechanisms could give some clues about potential strategies to prevent or revert programmed propensity to develop obesity, as well as may help in the identification of early biomarkers. Therefore, the main aim of this PhD Thesis has been: ***To characterize in rats the programming effects of moderate maternal energy restriction during pregnancy or lactation on the metabolic health of their offspring in terms of obesity and related metabolic alterations, as well as to identify new preventing strategies against programmed obesity and early biomarkers of metabolic health.***

We have characterized an animal model previously described to exhibit higher propensity to develop obesity and related metabolic alterations — the offspring of rat dams exposed to moderate food restriction during gestation (CRG) — to find out some of the potential mechanisms underlying their negative metabolic outcomes. Expression levels of key energy homeostasis-related genes in the hypothalamus and adipose tissue, as well as the measurement of some circulating parameters, showed that these animals were programmed, already from early stages of life, for a lower capacity to respond to insulin and to central leptin action. This could explain the hyperphagia observed in these animals (both genders) and the higher body weight occurring particularly in males. Some of these programmed metabolic disturbances, such as the impaired insulin and leptin sensitivity, and the increased systolic blood pressure, characteristic of CRG animals, were reverted by enhancing hepatic fatty acid oxidation at early ages, through adeno-associated virus (AAV)-mediated gene transference of the cDNA of *Cpt1am* (encoding for a permanently active form of CPT1A insensitive to its physiological inhibitor malonyl-CoA). AAV-*Cpt1am* injection in CRG animals was also able to ameliorate inflammatory state and restored the locomotive activity that was diminished in these animals in comparison to their controls.

Unlike calorie restriction during gestation, we show here that moderate calorie restriction in rat dams during lactation protects their offspring (CRL) against diet-induced obesity and related metabolic alterations, such as dyslipidemia, insulin resistance and hyperleptinemia. This condition during lactation determines early changes in WAT and liver, affecting lipogenic and oxidative capacity and increasing their sensitivity to the peripheral effects of leptin and insulin, which suggests a better control of energy metabolism. These adaptations occurring in early ages were partially maintained in adulthood and were particularly evident when animals were exposed to an obesogenic environment. Adult CRL animals showed gender-dependent changes at gene expression level in adipose tissue and hypothalamus, suggesting that males were more protected against HF-diet induced peripheral insulin resistance and also showed improved

capacity to respond centrally to leptin, while CRL females were programmed for a better sensitivity to the peripheral actions of leptin and to the central action of insulin. We used this animal model to identify early transcriptome-based biomarkers of improved metabolic health by whole-genome microarray analysis in peripheral blood mononuclear cells (PBMCs); these cells may be easily obtained by low invasive techniques and hence represent an attractive source for identifying biomarkers and to extrapolate such biomarkers from animals into humans. Concerning the factors potentially involved in the benefits of maternal calorie restriction during lactation, the analysis – by liquid chromatography with tandem mass spectrometry detection – of protein damage biomarkers in breast milk from CRL dams, suggests that the diminished content of glycation and oxidation free adducts during the second part of the lactating period, in comparison to control milk, could contribute to the health benefits observed in their adult offspring.



Efectos de una restricción energética moderada en las madres sobre la salud metabólica de las crías, en relación a la obesidad y las alteraciones metabólicas asociadas, e identificación de factores determinantes y biomarcadores tempranos.

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Resumen

Numerosas evidencias, procedentes tanto de estudios epidemiológicos en humanos como de modelos animales, indican que la salud materna y su estado nutricional durante la gestación y la lactancia pueden programar la propensión a desarrollar obesidad en la descendencia. Se están llevando a cabo grandes esfuerzos para entender los mecanismos moleculares responsables de dicha programación metabólica. La identificación de los mecanismos responsables podría dar ciertas pistas para el desarrollo de estrategias que permitan prevenir o revertir dicha propensión a desarrollar obesidad, así como también podría ayudarnos en la identificación de biomarcadores tempranos de salud metabólica. Por consiguiente, el principal objetivo de esta tesis doctoral ha sido: ***Caracterizar en ratas los efectos de una restricción energética materna moderada durante la gestación o la lactancia sobre la salud metabólica de la descendencia, en relación a la obesidad y las alteraciones metabólicas asociadas, así como también identificar nuevas estrategias de prevención frente la programación de la obesidad y nuevos biomarcadores tempranos de salud metabólica.***

Hemos caracterizado un modelo animal, que previamente se había descrito que presentaba una mayor propensión a desarrollar obesidad y alteraciones metabólicas asociadas – las crías de ratas sometidas a una restricción calórica moderada durante la gestación (CRG) – para identificar algunos de los posibles mecanismos responsables de sus efectos negativos. Los niveles de expresión de genes claves relacionados con la homeostasia energética en el hipotálamo y en el tejido adiposo, así como el análisis de ciertos parámetros circulantes, revelaron que estos animales estaban programados, ya desde etapas tempranas de la vida, para una menor respuesta a la insulina y a la acción central de la leptina, en relación con los animales control. Esto podría explicar la hiperfagia observada en estos animales (en ambos sexos), y el mayor peso corporal que presentan, particularmente los machos. Algunas de estas alteraciones metabólicas, tales como la alteración de la sensibilidad a la insulina y a leptina, así como la elevada presión sistólica, característica de los animales CRG, se vieron revertidas al favorecer el incremento de la oxidación hepática de ácidos grasos, en edades tempranas, a través de la transferencia génica mediada por vectores virales adeno-asociados (AAV) del ADNc de la *Cpt1am* (que codifica para una forma permanentemente activa de la CPT1A, insensible a su inhibidor fisiológico malonil-CoA). La inyección de AAV-*Cpt1am* en animales CRG también fue capaz de mejorar el estado de inflamación y de restaurar la actividad locomotora que estaba disminuida en estos animales en comparación con los controles.

A diferencia de la restricción calórica durante la gestación, observamos que la restricción calórica moderada en ratas madre durante la lactancia, protege a su descendencia (CRL) frente al desarrollo de obesidad inducida por la dieta y el desarrollo de alteraciones metabólicas asociadas, tales como la dislipidemia, la resistencia a la insulina y la hiperleptinemia. Esta condición durante la lactancia determina cambios tempranos en el tejido adiposo y el hígado,

afectando la capacidad lipogénica y oxidativa, e incrementando la sensibilidad a la acción periférica de la insulina y la leptina, lo que sugiere un mejor control del metabolismo energético. Dichas adaptaciones, que ocurrieron en edades tempranas, se mantuvieron parcialmente en edad adulta y fueron particularmente más evidentes cuando los animales fueron expuestos a un ambiente obesogénico. Los animales CRL adultos mostraron cambios a nivel de expresión génica en el tejido adiposo y en el hipotálamo que fueron dependientes del sexo, sugiriendo que los machos estaban más protegidos frente a la resistencia periférica a la insulina inducida por una dieta hiperlipídica, así como también mostraron una capacidad mejorada para responder a la leptina a nivel central; en cambio, las hembras CRL estaban programadas para una mejor sensibilidad a la acción periférica de la leptina y a la acción central de la insulina. Utilizamos este modelo animal para identificar marcadores tempranos de transcripción indicadores de salud metabólica mejorada mediante el análisis por microarray de células mononucleares de sangre periférica (PBMCs); estas células se pueden obtener fácilmente mediante técnicas poco invasivas por lo que representan una fuente atractiva para la identificación de biomarcadores y para extrapolar tales biomarcadores de animales a humanos. Con respecto a los factores que podrían estar potencialmente implicados en los efectos beneficiosos de la restricción calórica durante la lactancia, el análisis – por cromatografía líquida con sistema de detección por espectrometría de masas en tandem – de biomarcadores de daño proteico en la leche de madres CRL, sugiere que el menor contenido de aductos libres de glicación y oxidación durante la segunda mitad de la lactancia, en comparación con la leche de madres control, podría contribuir a explicar los efectos beneficiosos observados en las crías de estos animales en edad adulta.



Efectes d'una restricció energètica moderada a les mares sobre la salut metabòlica de les cries, en relació a l'obesitat i les alteracions metabòliques associades, i identificació de factors determinants i biomarcadors primerencs.

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Resum

Nombroses evidències, procedents tant d'estudis epidemiològics en humans com de models animals, indiquen que la salut materna i el seu estatus nutricional durant la gestació i la lactància, poden programar la propensió de la descendència per desenvolupar obesitat. S'està duent a terme grans esforços per entendre els mecanismes moleculars responsables d'aquesta programació metabòlica. La identificació d'aquests mecanismes podria donar-nos certes pistes per al desenvolupament d'estratègies que permetin prevenir o revertir la propensió programada per al desenvolupament d'obesitat, així com també podria ajudar-nos en la identificació de biomarcadors primerencs de salut metabòlica. Per això, el principal objectiu d'aquesta tesis doctoral ha estat: ***Caracteritzar en rates els efectes d'una restricció energètica moderada a les mares durant la gestació o la lactància sobre la salut metabòlica de la descendència, en relació a l'obesitat i les alteracions metabòliques associades, així com també identificar noves estratègies de prevenció enfront a la programació de l'obesitat i nous biomarcadors primerencs de salut metabòlica.***

Hem caracteritzat un model animal, que prèviament se va veure que exhibia una major propensió a desenvolupar obesitat i alteracions metabòliques associades – les cries de rates sotmeses a una restricció calòrica moderada durant la gestació (CRG) – per identificar alguns dels possibles mecanismes responsables dels seus efectes negatius. Els nivells d'expressió de gens clau relacionats amb la homeòstasi energètica a l'hipotàlem i al teixit adipós, així com també l'anàlisi de certs paràmetres circulants, varen mostrar que aquests animals estaven programats, ja des d'etapes primerenques de la vida, per una menor resposta a la insulina i a la acció central de la leptina respecte dels animals control. Això podria explicar la hiperfàgia observada en aquests animals (en ambdós sexes), i el major pes corporal, que presenten particularment els mascles. Algunes d'aquestes alteracions metabòliques, com ara l'alteració de la sensibilitat a la insulina i a la leptina, així com l'elevada pressió sistòlica, característica dels animals CRG, es van veure revertides en afavorir l'increment de la oxidació hepàtica dels àcids grassos, a edats primerenques, a través de la transferència gènica mitjançant vectors virals adeno-associats (AAV) de l'ADNc de la *Cpt1am* (que codifica per una forma permanentment activa de la CPT1A, insensible al seu inhibidor fisiològic, el malonil-CoA). La injecció de AAV-*Cpt1am* en animals CRG també va ser capaç de millorar l'estat d'inflamació i va restaurar la activitat locomotora que estava disminuïda en aquests animals en comparació amb els controls.

A diferència de la restricció calòrica durant la gestació, observarem que la restricció calòrica moderada en rates mare durant la lactància, protegeix a la seva descendència (CRL) enfront del desenvolupament d'obesitat induïda per la dieta i el desenvolupament d'alteracions metabòliques associades a l'adultesa, com ara la dislipèmia, la resistència a la insulina i la hiperleptinèmia. Aquesta condició durant la lactància determina canvis primerencs en el teixit adipós i en el fetge, afectant la capacitat lipogènica i oxidativa, i incrementant la sensibilitat a

la acció perifèrica de la insulina i la leptina, el que suggereix un millor control del metabolisme energètic. Aquestes adaptacions en edats primerenques se varen mantenir parcialment en edat adulta, i varen ser particularment més evidents quan els animals es varen exposar a un ambient obesogènic. Els animals adults CRL varen mostrar canvis en els nivells d'expressió gènica en el teixit adipós i a l'hipotàlem, que varen ser dependents del sexe, suggerint que els mascles estaven més protegits enfront a la resistència perifèrica a la insulina induïda per una dieta hiperlipídica, així com també mostraren una capacitat millorada per respondre a la leptina a nivell central; en canvi, les femelles CRL estaven programades per a una millor sensibilitat a l'acció perifèrica de la leptina i a la acció central de la insulina. Utilitzarem aquest model animal per a la identificació de biomarcadors primerencs de transcripció, indicadors de salut metabòlica millorada, mitjançant l'anàlisi per microarray en cèl·lules mononuclears de sang perifèrica (PBMCs); aquestes cèl·lules se poden obtenir fàcilment mitjançant tècniques poc invasives, pel que representen una font atractiva per a la identificació de biomarcadors i per extrapolar tals biomarcadors d'animals a humans. Pel que fa als factors que podrien estar potencialment implicats en els efectes beneficiosos de la restricció calòrica materna durant la lactància, l'anàlisi – per cromatografia líquida amb sistema de detecció per espectrometria de masses *en tadem* – de biomarcadors de dany proteic a la llet de mares CRL, suggereix que el menor contingut d'adductes lliures de glicació i d'oxidació durant la segona mitat de la lactància, en comparació amb la llet de mares control, podria contribuir a explicar els efectes beneficiosos observats en les cries d'aquests animals en edat adulta.

List of original articles

The present Doctoral Thesis is based on the following seven original research manuscripts:

1. Palou M., Konieczna J., Torrens JM., Sánchez J., Priego T., Fernandes ML., Palou A. and Picó C. **Impaired insulin and leptin sensitivity in the offspring of moderate caloric-restricted dams during gestation is early programmed.** J Nutr Biochem, 23:1627-39, 2012
2. Torrens JM., Orellana-Gavaldà JM., Palou M., Sánchez J., Herrero L., Picó C., Serra D. and Palou A. **Enhancing hepatic fatty acid oxidation as a strategy for reversing metabolic disorders programmed by maternal undernutrition during gestation.** Cell Physiol Biochem, 33:1498-515, 2014
3. Palou M., Priego T., Sánchez J., Torrens JM., Palou A. and Picó C. **Moderate caloric restriction in lactating rats protects offspring against obesity and insulin resistance in later life.** Endocrinology, 151:1030-41, 2010
4. Palou M., Torrens JM., Priego T., Sánchez J., Palou A. and Picó C. **Moderate caloric restriction in lactating rats programs their offspring for a better response to HF diet feeding in a sex-dependent manner.** J Nutr Biochem, 22:574-84, 2011
5. Torrens JM., Konieczna J., Palou M., Sánchez J., Picó C. and Palou A. **Early biomarkers identified in a rat model of a healthier phenotype based on early postnatal dietary intervention may predict the response to an obesogenic environment in adulthood.** J Nutr Biochem, 25:208-18, 2014
6. Konieczna J., Sánchez J., van Schothorst EM., Torrens JM., Bunschoten A., Palou M., Picó C., Keijer J. and Palou A. **Identification of early transcriptome-based biomarkers related to lipid metabolism in peripheral blood mononuclear cells of rats nutritionally programmed for improved metabolic health.** Genes Nutr, 9:366, 2014
7. Torrens JM., Palou M., Shaheen F., Pico C., Palou A., Rabbani N. and Thornalley PJ. **Moderate maternal calorie restriction in lactating rats affects markers of protein damage by glycation and oxidation in breast milk.** To be submitted.

My contribution in each manuscript was:

Manuscript 1: I performed the RNA extraction and the gene expression analysis of selected genes in the hypothalamus and rWAT, and the analysis of some circulating parameters. I also collaborated in the statistical analysis and approved the final version of the manuscript.

Manuscript 2: I carried out the animal experiment (with the collaboration of other authors for the injection of vectors) and performed all the analyses. I also analyzed all data, participated in the discussion, and wrote a first version of the manuscript.

Manuscript 3: I collaborated in the animal experiment. Moreover, I performed the RNA extraction and the analysis of the gene expression levels of leptin in mammary gland and WAT. I also collaborated in the data analysis and approved the final version of the manuscript.

Manuscript 4: I collaborated in the animal experiment. I also performed the RNA extraction and the analysis of the gene expression levels of selected genes in WAT, and I contributed in the analysis of some circulating parameters. I also collaborated in the data analysis. I read and approved the final version of the manuscript.

Manuscript 5: I carried out the animal experiment in collaboration with another author. I contributed in the performance of the oral fat tolerance test. I performed the measurement of some circulating parameters, the RNA extraction and the gene expression analysis in WAT and liver. I analyzed the data and wrote a first version of the manuscript.

Manuscript 6: I performed the animal experiment in collaboration with another author. I collaborated in the isolation of PBMCs from blood samples. I contributed in the measurement of some circulating parameters, in the RNA extraction from PBMCs and tissues, as well as in the gene expression analysis of selected genes in WAT and liver. I also collaborated in the data analysis. I read and approved the final version of the manuscript.

Manuscript 7: I carried out the animal experiment and the collection of maternal plasma and breast milk samples. I collaborated in the analysis of free amino acids and protein damage markers by using the technique of liquid chromatography with tandem mass spectrometry detection. I also collaborated in the analysis of the data, discussion of the results and wrote a first version of the manuscript.

Chapter I.

Introduction

Introduction

1. Obesity

According to World Health Organization (WHO), overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health. Clinicians and epidemiologist usually rely on body mass index (BMI) that is considered a simple index of weight-for-height and commonly used to classify overweight and obesity in adults (WHO·2013). BMI is defined as a person's weight in kilograms divided by the square of his height in meters (kg/m^2). The WHO defines: a BMI of 30 or more is obesity; and a BMI equal to or more than 25 is overweight. Nowadays, it is calculated that 400 million adults have a BMI exceeding $30 \text{ kg}/\text{m}^2$ and this number is forecast to rise to 700 million by 2030, especially in developed countries (Shaw, et al. 2010).

Overweight and obesity are the fifth leading risk for global deaths. At least 2.8 million adults die each year as a result of being overweight or obese (WHO·2013). It is due to the fact that obesity is a central factor in development of insulin resistance, Type 2 diabetes and metabolic syndrome, and all of which create an increased risk of cardiovascular disease (Rask-Madsen and Kahn 2012). Metabolic syndrome itself is characterized by central obesity plus a cluster of key risk factors of obesity-related disorders, including impaired glucose tolerance, diabetes mellitus, hypertension and dyslipidemia (Alexander, et al. 2003). The WHO defines that 44% of the diabetes burden and 23% of the ischemic heart disease burden are attributable to overweight and obesity (WHO·2013). Furthermore, a very worrisome fact is the hugely increased level of childhood obesity, that is rising with the same pattern that of the adult population. In the USA, 10% of preschool children are obese and half of these are thought to have impaired glucose tolerance (Rocchini 2002). As childhood obesity is a major risk factor for adult obesity, 20% incidence of childhood obesity portends a further increase in the prevalence of the also early appearance of Type 2 diabetes [reviewed by (Desai, et al. 2013)]. Therefore, obesity and its associated illnesses not only are a rising worldwide health problem, but they are also placing nowadays an enormous financial burden on society for the care and treatment of patients. It has been calculated that over the next 20 years, the healthcare costs attributable to obesity will rise to about 16% of the total of those costs in Western countries (Wang, et al. 2011). In this sense, many attempts have been done by researchers to understand the biology, aetiology and pathogenesis of obesity.

The pathogenesis of obesity is very complex. Evidences from twin studies and from adoption and family studies strongly support the concept that genes play a central role in the determination of BMI and, consequently, in the pathogenesis of obesity [reviewed by (Xia and Grant 2013)]. By 2011, nine loci have been recognized to be involved in monogenic forms of obesity (Choquet and Meyre 2011). Among these loci, those that encode for leptin, leptin receptor, proopiomelanocortin (*Pomc*) and melanocortin 4 receptor (*Mc4r*) are relevant because a single gene mutation in one of these loci, with a recessive or dominant mode of inheritance, has been associated to extremely severe phenotypes of obesity in childhood (Mutch and Clement 2006). However, genetic predisposition to obesity for most individuals has a polygenic basis, and hence, each single polygene makes a small contribution to the development of obesity. Concretely, until now, 58 loci have been described to contribute to polygenic obesity (Choquet and Meyre 2011). Among them, common genetic variants with replicable effects on obesity susceptibility are: catenin beta like (*Cttnb1l1*), fat mass and obesity associated (*Fto*), *Mc4r*, proprotein convertase subtilisin/kexin type 1 (*Pcsk1*) and insulin-induced gene 2

(*Insig2*). Particularly, the receptor variant with the amino acid isoleucine (wildtype: valine) at position 103 of the MC4R represents the first confirmed polygenic variant with an influence on body mass index. Moreover, variants in the first intron of the gene *Fto* confer the most pronounced polygenic effect on obesity (Hinney and Hebebrand 2008). Interestingly, recent studies in mice have demonstrated that obesity-associated noncoding sequences within *Fto* are functionally connected with the iroquois homeobox 3 gene (*Irx3*). This finding suggests that *Irx3* is a functional long-range target of obesity-associated variants within *Fto*, representing a novel determinant of body mass and composition (Smemo, et al. 2014).

However, over the past few years, obesity in developed countries has been increased too rapid to be accounted for only by genetics. Nowadays, it is known that the recent acceleration of obesity and overweight incidence is due to a complex interaction among behavioural, environmental, and genetic factors which ultimately lead to a chronic energy imbalance that favours energy accumulation and excessive weight gain. Obesity is positively associated with dietary patterns of western societies, such as increased fat intake, lower fibre consumption, increased hidden sugars in prepared foods, reduced amounts of unrefined sugars and an inadequate fruit and vegetable intake (Chantel, et al. 2002). Physical activity pattern also plays an important role in obesity. Sedentary behaviours, such as prolonged TV watching, video game playing, computer use, sedentary nature of many forms of work and increasing urbanization have been expanded rapidly in developed countries contributing to obesity development (Ogunbode, et al. 2011). Indeed, technological inventions have created many time- and labor-saving products. As a result, people have reduced the overall energy expenditure in their daily lives, which also contributes to obesity development (Verduin, et al. 2005).

In addition to the mentioned interacting factors that lead to obesity development, climbing evidence in human and animal models indicate that certain stimuli experienced during early life may have an important role programming the risk of obesity and its related metabolic disorders (Guilloteau, et al. 2009; Martin-Gronert and Ozanne 2013; Picó, et al. 2012). In particular, it has become apparent that early life environment, especially nutrition during critical periods of development, such as pregnancy and lactation, can have long-lasting effects programming the susceptibility of an individual to develop obesity and other features of the metabolic syndrome in adult life. Such programming causes permanent alterations in the development and organization of energy balance-related tissues and organs that leads to irreversible changes in the structure and function of the body influencing the propensity to obesity and related metabolic alterations in adult life (Martin-Gronert and Ozanne 2013).

Therefore, knowing the consequences of obesity for quality of life, morbidity and health costs, it becomes very important to understand the aetiology of obesity as well as to elucidate the mechanisms underlying the threatening increase in obesity rates among the adult population, and even among children. In this regard, identification of these mechanisms could be the basis for the description of early biomarkers which may serve as biological indicators of the propensity of an individual to develop obesity later in life. Moreover, identification of the mechanisms involved in obesity development could be the tool for developing new strategies to prevent or revert the increased risk for obesity.

2. Energy balance regulation

As described above, obesity results from a prolonged imbalance between energy intake and energy expenditure, in favour of energy intake. Energy homeostasis is defined as a coordinated balance among food intake, energy expenditure and storage, which maintain stability of the

metabolic state. It is under the control of an integrated system that has the capacity to rapidly respond to metabolic changes.

Energy homeostasis is mainly regulated by the central nervous system (CNS), especially by the hypothalamus that plays an important role in central regulation of appetite and body composition (Brenseke, et al. 2013). In addition, a set of peripheral tissues, such as liver, adipose tissue and skeletal muscle, has also been described as metabolic tissues with an important role in global energy balance regulation under the control of the CNS (Lee, et al. 2003; Pang and Han 2012).

2.1. Main tissues involved in maintenance of energy homeostasis

2.1.1. Hypothalamus

The hypothalamus is the portion of the brain located below the thalamus, just above the brainstem, that contains a number of small nuclei that produce neuropeptides involved in numerous physiological and behavioural functions (**Figure 1A**). Among them, one of the most important functions of the hypothalamus is to link the nervous system to the endocrine system via the hypophysis. This connection allows the hypothalamus to be informed about the nutritional, energetic and environmental status of the body through peripheral and central orexigenic or anorexigenic messages (Valassi, et al. 2008).

Experimental efforts have been made to understand how the brain regulates energy homeostasis and how impaired brain functions contribute to the pathogenesis of obesity, diabetes and related metabolic disorders. The specific hypothalamic structures that are directly involved in energy homeostasis were identified when a set of systematic lesion experiments were performed in rats (Brobeck 1946). In several experiments, various hypothalamic nuclei, including the hypothalamic ventromedial (VMN), paraventricular (PVN) and dorsomedial (DMN) nuclei, were destroyed. The lesion of these nuclei induced hyperphagia and obesity, whereas lesions in the lateral hypothalamus (LH) led to hypophagia. These studies led to the proposal of a “dual center model” that identified the hypothalamic VMN as the “satiety center”, and the LH as the “hunger center” [reviewed by (Gao and Horvath 2008)].

The hypothalamic arcuate nucleus (ARC) is located at the base of the hypothalamus around the third ventricle and is considered a critical region in food intake and energy homeostasis regulation due to various reasons (Elmqvist, et al. 1999). Firstly, their neurons, known as “first-order neurons” have their nerve endings anatomically placed in close proximity to fenestrated capillaries at the base of the hypothalamus, giving them access to humoral signals that are restricted from other regions of the brain. Indeed, they respond rapidly to fluctuations in nutrients and metabolic hormones. Finally, they project broadly to the brain and periphery both directly as well as indirectly [reviewed by (Gao and Horvath 2008)].

ARC neurons are separated in two populations of neurons with opposite effects on feeding behaviour. In this sense, there is a family of anorexigenic neurons that co-express cocaine and amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC), which is the precursor for many active neuropeptides including melanocyte-stimulating hormone (α -MSH). These, in turn, reduce food intake and body weight as well as increase energy expenditure in animals and humans by acting on melanocortin receptor subtypes 3 and 4 (MC3R and MC4R, respectively), found to be abundant in the ARC, PVN, LH and DMN. In contrast, there is a family of orexigenic neurons that co-express neuropeptide Y (NPY) and Agouti-related peptide (AgRP). NPY potently stimulates food intake and reduces energy expenditure. AgRP acts as a

natural antagonist of MC3R and MC4R, and hence reduces the anorectic effect of α -MSH [reviewed by (Gao and Horvath 2008)].

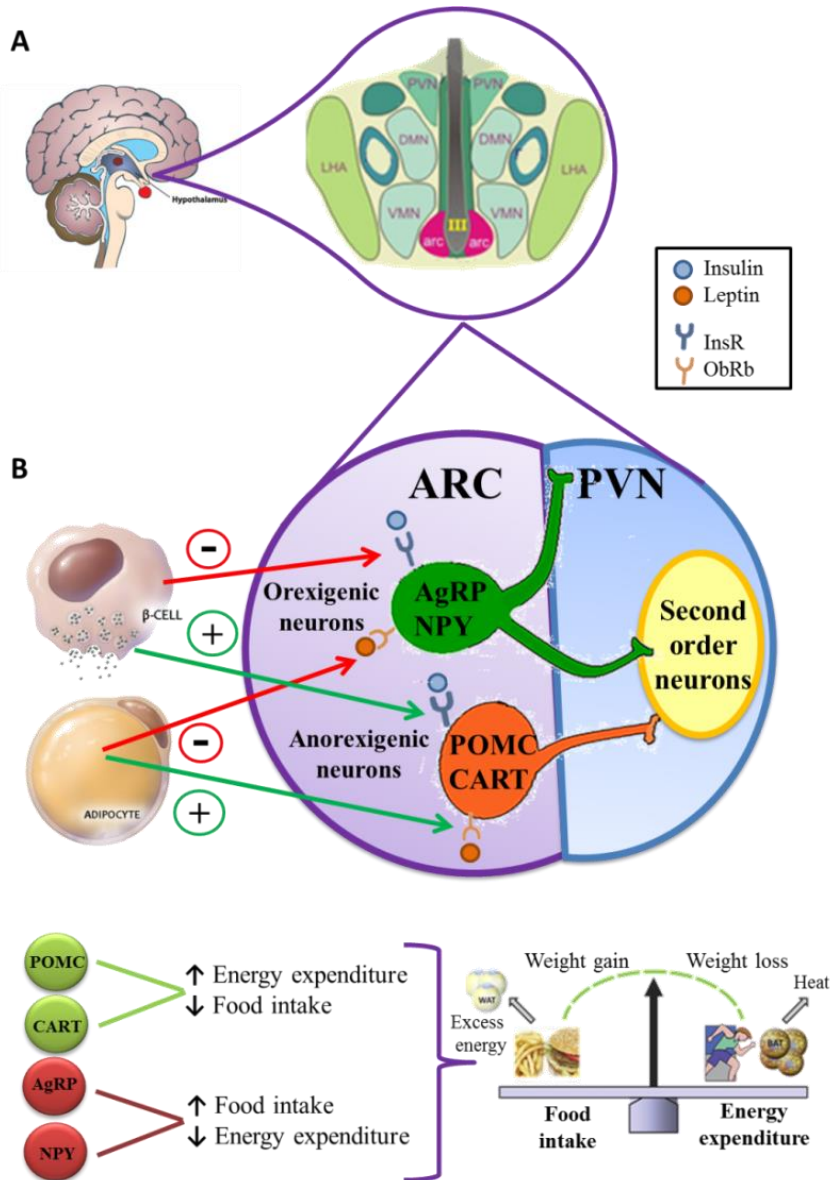


Figure 1. A. Anatomical localization of the arcuate nucleus (ARC) and the surrounding hypothalamic nuclei: DMN, dorsomedial nucleus; LHA, lateral hypothalamic area; PVN, paraventricular nucleus and VMN, ventromedial nucleus. **B.** Schematic diagram of the ARC. Two populations of “first-order” neurons, with opposite effects on food intake regulation and energy expenditure, are located in the ARC. Anorexigenic neurons, which release the neuropeptides Proopiomelanocortin (POMC) and Cocaine and Amphetamine-regulated transcript (CART); and the orexigenic neurons, which release the neuropeptides Agouti-related peptide (AgRP) and the Neuropeptide Y (NPY). The axons of these neurons project to “second-order” neurons, located mainly in PVN and other hypothalamic regions. Both insulin and leptin are secreted into the bloodstream in direct proportion to body fat stores, and both interact with neurons in the ARC. Insulin and leptin activate POMC and CART neurons via their respective receptors, insulin receptors (InsR) and leptin receptor (ObRb). This results in the activation of downstream catabolic neurons which in turn reduce food intake and increase energy expenditure. Conversely, the NPY and AgRP expressing neurons are inhibited by insulin and leptin, reducing the secretion of NPY and AgRP which potently stimulate food intake and reduce energy expenditure. Adapted from (Niswender and Schwartz 2003).

The axons of ARC neurons project to “second-order” neurons located in various parts of the brain, such as PVN and LH, where there are substantial numbers of MCR3 and MCR4. The

projection from the ARC to the PVN is important for the regulation of neurons that produce the anorexigenic substances, such as corticotropin and thyrotropin releasing hormones (CRH and TRH respectively), and for the modulation of sympathetic activity, both of which are significant mechanisms in energy metabolism. The projection from the ARC to the LH is important for the production of orexigenic molecules, such as melanin-concentrating hormone (MCH) and orexins (Valassi, et al. 2008).

Hypothalamus, and particularly the hypothalamic ARC structure, integrates the peripheral information of hormones (insulin, ghrelin and leptin) and nutrients, such as glucose, to maintain energy balance (Breton 2013; Morton, et al. 2006). Concretely, leptin and insulin act at hypothalamic level by binding to its respective receptors, the long form of leptin receptor (ObRb) and insulin receptor (InsR) respectively, which have been localized in the ARC (Baskin, et al. 1998). Both receptors are linked to the phosphatidylinositol 3-kinase (PI3K) pathway and reduce the expression and release of the orexigenic peptides (NPY and AgRP) and activate anorexigenic peptides (CART and POMC) (**Figure 1B**).

In addition, other hypothalamic nuclei, especially the PVN, may in turn also modulate energy expenditure such as lipolysis and/or thermogenesis in adipose tissue, via the sympathetic autonomic nervous system (Fliers, et al. 2003).

2.1.2. White adipose tissue

White adipose tissue (WAT) is the major body's energy storage. The main form of energy storage is represented by high amounts of triglycerides (TGs) in adipocyte lipid droplets (Daval, et al. 2006). The stored TGs are derived from fatty acids which can be either taken up from the diet or synthesized within adipocytes by *de novo* synthesis from non-lipid substrates (Fliers, et al. 2003). In order to take up fatty acids from plasma, adipocytes synthesize a specific insulin-stimulated enzyme called lipoprotein lipase (LPL), which is exported to the luminal side of vascular endothelium where it can hydrolyse TG-rich lipoproteins such as chylomicrons and very low density lipoproteins (VLDLs) to yield fatty acids and free glycerol. Fatty acids enter into the adipocytes through transporters and are re-esterified with glycerol phosphate to form TGs stored in a single lipid droplet in white adipocytes (Daval, et al. 2006). In addition, glucose also provides the glycerol backbone for TG (Aguilera, et al. 2008). Fatty acids can also be *de novo* synthesized from glucose, however, the rate of synthesis of fatty acids from glucose in humans is lower than in rodents (Aguilera, et al. 2008). When food is available, via lipogenic pathways, adipocytes store excess of energy as TGs to guarantee the survival in periods of increased energy expenditure or decreased energy availability. Conversely, when energy is needed, TGs are hydrolysed into fatty acids and glycerol, which are exported back into the blood, via activation of lipolytic pathways mainly driven by noradrenergic innervation (Breton, 2013). The key enzymes of norepinephrine-stimulated lipolysis are adipose tissue triglyceride lipase (ATGL), which is the main protein involved in the catalysis of the initial step in TG hydrolysis in adipocyte lipid droplets, and hormone-sensitive lipase (HSL), which hydrolyses stored TGs to free fatty acids as well as several other lipids stored in WAT. Particularly, HSL is regulated by numerous factors and hormones through several mechanisms, including reversible phosphorylation via noradrenergic innervation. This phosphorylation stimulates adenylate cyclase and increases intracellular levels of cyclic adenosine monophosphate (cAMP). Then, cAMP activates protein kinase A (PKA) which in turn phosphorylates and activates HSL. Although ATGL works in conjunction with HSL, its activity is not dependent on PKA phosphorylation (Daval, et al. 2006).

In addition to its functions related to energy storage and release, WAT plays a key role as an endocrine organ controlling energy homeostasis and substrate partitioning (Daval, et al. 2006). WAT is involved in steroid hormone metabolism and glucocorticoid metabolism. Moreover, WAT produces adipocytokines, such as leptin and adiponectin; other hormones, like resistin; appetite-regulating related peptides, and proteins from the renin-angiotensin system. Among all of them, resistin has been linked with insulin resistance induction while adiponectin has been associated with insulin sensitivity enhancement (Fliers, et al. 2003). Some of these circulating factors act in an autocrine/paracrine manner as adipocyte lipid metabolism regulators, while some of them act as peripheral endocrine signals to regulate energy homeostasis at hypothalamic level, as leptin (Breton 2013).

Finally, adipose tissue also secretes other proteins that may be of interest in view of the association between obesity and cardiovascular risk inflammatory. Among them, there are cytokines such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF α), and several coagulation and complement factors, such as plasminogen activator inhibitor-1 (the key modulator of the fibrinolytic pathway), tissue factor (the most powerful activator of the coagulation system) and fibrinogen-angiopoietin-related protein (a regulator of angiogenesis and modulator of tumorigenesis) (Fliers, et al. 2003; Trayhurn and Beattie 2001). Concerning, IL-6 and TNF α , these cytokines are involved in systemic inflammation and also appears to participate in the induction and maintenance of the subacute inflammatory state associated with obesity. Moreover, TNF α is overproduced in fatty liver and participates in the development of insulin resistance (Shoelson, et al. 2006) and IL-6 can also favour insulin resistance in insulin sensitive tissues.

2.1.3. Liver

Liver is recognized as a metabolic, endocrine and exocrine organ that is responsible for many vital functions, including formation of bile salts, excretion of bilirubin and cholesterol, blood detoxification and purification, immunological processes and production of critical coagulation factors, serum proteins and hormones. In addition, due to the fact that all the blood coming from the digestive system passes through the hepatic portal vein, liver also plays an important role in storage of energy and nutrients obtained from blood, as well as in the metabolism of lipids, proteins and carbohydrates. In this sense, alterations in the ability of the liver to properly regulate lipid metabolism and glucose homeostasis are the heart of the deleterious consequences of obesity and insulin resistance (Brock and Dorman 2007).

Regarding lipid metabolism and transport, liver is considered the most dynamic organ of the body and the key site of metabolic integration (Brock and Dorman 2007). Liver is able to acquire fat and cholesterol from the diet. Concretely, dietary TGs and cholesterol are transported from the intestine in the form of chylomicrons. Rapidly, the TGs in the lipid core of the chylomicrons are hydrolysed at the capillary surface by the enzyme LPL, which yields smaller particles known as chylomicrons remnants. The released free fatty acids are used either for storage in adipose tissue or for oxidation in other tissues, while dietary cholesterol is transported in the chylomicrons remnants to liver (Gotto 1990; Sandhofer 1994). Cholesterol and TG are also synthesized in the liver and then secreted into the blood in the form of VLDL. TGs from VLDL particles are also hydrolysed by the enzyme LPL, to yield intermediate density lipoproteins (IDL) which are either taken up by the liver or further catabolized to low-density lipoprotein (LDL). These LDL are bound and taken up by specific receptors in the liver and many other tissues; by this pathway, cholesterol is transported from the liver to peripheral tissues (Sandhofer 1994). Moreover, when hepatic glycogen stores are in a state of excess, liver

synthesises *de novo* fatty acids by deviation of acetyl-CoA to the fatty acid synthase complex (FASC), located within the cytoplasm of hepatocytes. Hepatic fatty acids are stored in the form of TGs, by esterification of acetyl-CoA with glycerol-3-phosphate. Once formed, these TGs are packaged to form VLDL, which are transported out of the liver and back to peripheral adipocytes. In general, there is an equilibrium of fatty acids between the peripheral adipocytes and the liver without any appreciable accumulation of lipids [reviewed by (Brock and Dorman 2007)].

Furthermore, liver influences strategically glucose homeostasis through a delicate balance between hepatic glucose production through glycogenolysis and gluconeogenesis during the fasted state, and hepatic glucose uptake, storage and utilization in the fed state. Transitions between the two states in liver are possible due to the joined physiological action of insulin and glucose promoting the expression of genes normally induced during the fed state and down-regulating the expression of genes normally activated during the fasted state (Collier and Scott 2004). On the one hand, representative genes expressed during the fed state are: glucokinase (*Gck*), liver pyruvate kinase (*Pkl*), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (*Pfkfb2*), acetyl-coenzyme A carboxylase (*Acc*), and fatty acid synthase (*Fasn*). On the other hand, the representative genes that are activated during the fasted state are: phosphoenolpyruvate carboxykinase (*Pck1*), fructose-1,6-bisphosphatase (*Fbp1*), and carnitine palmitoyl transferase 1 and 2 (*Cpt1* and *Cpt2*) [reviewed by (Collier and Scott 2004)]. The transcription factor, peroxisome proliferator-activated receptor alpha (PPAR α) plays an important role in liver regulating directly the expression of genes involved in fatty acid uptake, such as the gene encoding the fatty acid transport protein (*Fatp*) and fatty acid binding protein (*Fabp*). In addition, PPAR α also regulates the genes involved in plasma VLDL TG hydrolysis, such as *Lpl*, and also β -oxidation-related genes, such as *Cpt1a* and acyl-CoA oxidase 1 (*Acox1*) (Lee, et al. 2003; Martin, et al. 2009; McIntosh, et al. 2013).

While in the fed state the fuel source are carbohydrates and fats, in the fasted state, the main fuel source shift to fats. Fatty acids that were stored during feeding are released from the adipocyte and taken up by the liver. There, they are either reesterified to TGs and assembled into VLDL or broken down through mitochondrial hepatic fatty acid oxidation (FAO) and used to generate ketone bodies (Lee, et al. 2003).

Mitochondrial FAO represents a crucial process in energy metabolism. Hepatic FAO is tightly regulated by interaction between the key enzyme CPT1A and the ACC via intermediate malonyl-CoA (McGarry, et al. 1977). Malonyl-CoA, derived from glucose metabolism, is the first intermediate in lipogenesis and regulates FAO by inhibiting CPT1A. CPT1A is localized in the outer mitochondrial membrane and expose its active site at the cytosolic face of the mitochondria (Schreurs, et al. 2010). CPT1A regulates the transfer of long-chain acyl-CoAs from the cytosol into the mitochondria, where they are oxidized. Considering this and taking into account that obesity results from a chronic imbalance between energy intake and energy expenditure, which leads to fat accumulation in lipid deposits, strategies able to tilt the energy balance towards FAO could be considered as potential ways to treat or prevent obesity and related metabolic disorders. In this regard, genetic studies that increased hepatic FAO have been already developed. On the one hand, *in vitro* studies showed that hepatocytes transduced with adeno-associated viruses (AAV) encoding carnitine palmitoyltransferase 1a (*CPT1a*) increased the rate of β -oxidation that, in turn, led a decrease in TG content in liver (Stefanovic-Racic, et al. 2008). In other studies, the gene of *Cpt1a* was transferred into obese mice by injecting AAV vectors into the tail vein. This led to a nonimmunoreactive long-term increase in lipid oxidation. Concretely, Orellana-Gavaldà et al. used a mutant but permanently active form of CPT1A, the CPT1AM, which is insensitive to malonyl-CoA and therefore leads to a

permanent increase in the rate of FAO, independently of the glucose-derived malonyl-CoA levels. The results of this study showed that an increased hepatic FAO through AAV-mediated gene transference of *Cpt1a* or *Cpt1am* reduced obesity-induced hepatic steatosis, weight gain, inflammation, diabetes, glucose levels and insulin resistance in mice consuming a high-fat (HF) diet (Orellana-Gavaldà, et al. 2011).

2.1.4. Skeletal muscle

Skeletal muscle is a major mass peripheral tissue that accounts for approximately 40% of the total body mass. Skeletal muscle serves for overlapping functions, such as movement, posture, stability, communication, heat production, and cold tolerance (Smith and Muscat 2005). As the motor of the body, muscle requires considerable amounts of energy in the form of ATP. Muscle can burn energy faster than can be produced within the cell necessitating a buffer system that uses creatine kinase (CKM) to transfer a high-energy phosphate from phosphocreatine stores to ADP to form ATP. For short bursts of activity, skeletal muscle relies upon glycolysis for ATP production, which is the primary form of energy obtaining (Smith, et al. 2013).

In addition to these functions, skeletal muscle is being recognized also as an endocrine organ because expresses and releases into the circulation several cytokines that exert their effect in other parts of the body (Tomas, et al. 2004). These cytokines have been coined “myokines” and are for example, IL-6 and IL-15 (Smith and Muscat 2005). Concretely, IL-6 is able to sense the energy demands in muscle and signal to central and peripheral organs to maintain energy supply. In this sense, sustained and intense exercise induces skeletal muscle to express and release IL-6 into the circulation, especially when glucose and glycogen stores are low. Then, IL-6 stimulates 5' adenosine monophosphate-activated protein kinase (AMPK) activity which activates lipolysis in adipose tissue, inhibits TNF α , and also improves insulin sensitivity [reviewed by (Smith and Muscat 2005)].

Skeletal muscle is also a metabolic flexible tissue and has an essential role in energy balance because is the primary tissue of insulin stimulated glucose uptake, disposal, and storage in form of glycogen, even four-fold the glycogen content of liver. Moreover, skeletal muscle regulates cholesterol efflux and highly influences metabolism via modulation of circulating and stored lipid flux (Smith and Muscat 2006).

Fatty acids and glucose are the main energy sources in muscle. Particularly, during initial aerobic exercise, skeletal muscle uses as energy fuel the stored muscle glycogen which is broken down back into glucose via the enzyme glycogen phosphorylase; then, glucose is broken down into pyruvate during glycolysis providing the required energy in the form of ATP. If exercise continues, then, glucose from glycogen and stored muscle TGs become important energy substrates, and muscle must utilize the more efficient oxidative phosphorylation process. In this regard, pyruvate conversion to acetyl-CoA by pyruvate dehydrogenase allows progression through the tricarboxylic acid (TCA) cycle and subsequent oxidative phosphorylation to obtain energy. Concerning stored muscle TGs, the enzyme HLS is responsible for triglyceride breakdown to free fatty acids in muscle; the released free fatty acids are then transported into the mitochondria to undergo β -oxidation and produce acetyl-CoA and NADH (Smith, et al. 2013). If exercise is prolonged over long periods, then fatty acids and lipid mobilization from other tissues are increasingly needed (Smith and Muscat 2005).

Therefore, given the fact that skeletal muscle can utilize both carbohydrate and lipid energy substrates and considering the relative mass of the tissue, it is hardly surprising that normal or

abnormal skeletal muscle metabolism has an important impact on insulin sensitivity, on blood lipid profile and on the pathogenesis of metabolic diseases (Smith and Muscat 2006).

2.2. Main hormones involved in maintenance of energy homeostasis

A growing body of evidence suggest that hormones produced by adipose tissue, such as the adipocytokine leptin, and other hormones, such as the pancreatic insulin, play a critical role in the regulation of energy intake, energy expenditure and lipid and carbohydrate metabolism (Havel 2004). Understanding the biological actions, regulation and signalling mechanisms of these hormones will provide insights into the pathogenesis and treatment of obesity and its related metabolic disorders, particularly insulin and leptin resistance.

2.2.1. Leptin

Leptin, the *ob* gene product, is a protein of 167 amino acids. It is synthesized mainly by white adipocytes, but low levels are also produced in the stomach (Cinti, et al. 2001), placenta (Masuzaki, et al. 1997), skeletal muscle (Wang, et al. 1998) and mammary epithelium (Smith-Kirwin, et al. 1998). The concentrations of leptin in adipose tissue and plasma are closely parallel to the mass of adipose tissue and adipocyte size and TG content. Thus, leptin increases in obesity and falls with weight loss (Ahima, et al. 2000).

Circulating leptin plays a pivotal role in control of body weight and energy metabolism by communicating the body fuel availability to the CNS to adapt energy homeostasis (Pang and Han 2012). Leptin is secreted into blood, enters the brain in proportion to its plasma levels and reaches neuronal targets via a saturable transport mechanism across the blood-brain barrier (Banks, et al. 1996). Once in the CNS, leptin binds its receptors, ObRb, in the ARC and regulates energy homeostasis by stimulating the expression of the anorexigenic neuropeptides (POMC and CART) and by inhibiting the expression of the orexigenic neuropeptides (NPY and AgRP). These ARC neurons project on to “second-order” neurons, in the PVN and DMN (Schwartz, et al. 2000), to modulate glucose homeostasis, suppress feeding, stimulate thermogenesis and enhance lipid oxidation and insulin sensitivity in peripheral organs (Ahima and Lazar 2008). This leads to prompt appropriate regulation of food intake and energy expenditure processes and help our body to maintain the amount of fat stores within a certain range (Martin-Gronert and Ozanne 2013).

Concerning leptin signal transduction pathway (**Figure 2**), when it binds to the extracellular domain of the leptin receptor, activates Janus-activated Tyrosine Kinase 2 (JAK2) which leads to autophosphorylation of tyrosine residues on JAK2 and phosphorylation of Tyr⁹⁸⁵, Tyr¹⁰⁷⁷, and Tyr¹¹³⁸ on leptin receptor. Phosphorylation of Tyr¹¹³⁸ mediates the activation and nuclear translocation of the signal transducer and activator of transcription 3 (STAT3), which induces the transcription of neuropeptides in the hypothalamus as well as of the suppressor of cytokine signalling 3 (SOCS3), which terminates leptin signalling (Ahima and Lazar 2008). Leptin also activates PI3K, which induces the synthesis of phosphatidylinositol-3,4,5-triphosphate (PIP3) from phosphatidylinositol-4,5-biphosphate (PIP2). Accumulation of PIP3 leads to 3-phosphoinositide-dependent protein kinase 1 (PDK1) activation, and thus activates protein kinase B (PKB, also known as AKT). Activation of both pathways has been shown to be required for proper regulation of energy and glucose homeostasis (Konner and Bruning 2012)

In addition to its central anorexigenic actions, leptin also has a peripheral role as a mediator of energy expenditure acting in a paracrine/autocrine way in a range of cell types (Margetic, et al.

2002). Particularly, leptin receptors have been found in liver, adipose tissue, heart, kidneys, lungs, small intestine, pituitary cells, testes, ovaries, spleen, pancreas and adrenal gland [reviewed by (Margetic, et al. 2002)]. Then, when leptin binds to its cell-membrane receptors, induces the phosphorylation of STAT3 via the same signal transduction pathway as in the hypothalamus. Hence, once STAT3 is activated, it penetrates into the nucleus and regulates the transcriptional activity of leptin-controlled genes in the target tissues (Aguilera, et al. 2008).

Leptin is currently considered the major liporegulatory hormone, maintaining the normal intracellular lipid homeostasis (Aguilera, et al. 2008). Leptin avoids accumulation of TGs outside the adipose tissue, increasing glucose uptake, glycogen synthesis and lipid partitioning at skeletal muscle (Ahima and Lazar 2008; Houseknecht, et al. 1998). Particularly, leptin limits accumulation of TGs in muscles and liver, at least in part, stimulating FAO through AMPK activation (Daval, et al. 2006). In this sense, AMPK, activated by leptin, phosphorylates the enzyme malonyl-CoA decarboxylase and the lipogenic enzyme ACC, which is involved in the initial phase of fatty acid synthesis. These phosphorylations result in the activation of malonyl-CoA decarboxylase and the inhibition of ACC, leading to decreased levels of malonyl-CoA. Inhibition of malonyl-CoA formation, in turn, increases the activity of the CPT1, because malonyl-CoA is the physiological allosteric inhibitor of the CPT1. This results in the inhibition of lipid synthesis and in the enhancing of the mitochondrial FAO (Ahima and Lazar 2008).

In addition, leptin also down-regulates the activity of lipogenic transcription factors, mainly peroxisome-proliferator-activated receptor-gamma2 (PPAR γ 2), which is essential for adipogenesis, and, in liver cells, the sterol-regulatory-element-binding protein (SREBP1c), which regulates cholesterol and lipid synthesis (Sampath and Ntambi 2006). Hence, down-regulation of these transcription factors results in a decreased expression of the *Acc* and *Fasn*, and an increased expression of key enzymes involved in FAO, such as *Acox* and *Cpt1*, particularly in adipose tissue. Moreover, AMPK activation in skeletal muscle by leptin also appears to be related with enhancement of the intracellular expression of PPAR γ co-activator 1 α (PGC-1 α), which facilitates glucose entry and ensures its effective utilization by increasing the expression of genes involved in oxidative phosphorylation. In addition, PGC-1 α has emerged as a key regulator of mitochondrial biogenesis along with AMPK [reviewed by (Roman, et al. 2010)].

Furthermore, another physiological effect of leptin is the inhibition of insulin secretion in pancreatic β -cell by activation of cyclic nucleotide phosphodiesterase 3B through the PIP3 signalling transduction pathway (Zhao, et al. 1998). In addition, leptin also exerts multiple autonomic and cardiovascular effects, including activation of sympathetic nerve activity, increasing levels of endothelium-derived nitric oxide, and promoting angiogenesis [reviewed by (Konner and Bruning 2012)].

All in all, leptin is a peripheral hormone that acts centrally, at hypothalamic level, as an anorexigenic hormone modulating feeding patterns and energy expenditure. Besides of its central effects, leptin also acts peripherally regulating the entire body weight, and has antisteatotic effects by maintaining lipid homeostasis (Huan, et al. 2003).

Leptin during the perinatal period

As above mentioned, the main source of leptin in adults is the adipose tissue and its principal role is energy balance regulation. In neonates, serum leptin levels vary dramatically, because their mothers are contributing to the supply of leptin through the placenta during the gestational period and through breast milk during lactation (Cinti, et al. 2001; Palou and Picó 2009; Pico,

et al. 2011). However, neonatal leptin is not fully functional in the regulation of energy balance but has basically a developmental role, acting as an important neurotropic factor that facilitates the development of the neuronal hypothalamic circuits that govern metabolism, adipose tissue distribution and food intake (Keen-Rhinehart, et al. 2013; Remmers and Delemarre-van de Waal 2011).

At birth, circulating leptin levels in rodents are very low. However, several studies have shown that there is a surge in plasma leptin concentration, with a 5- to 10-fold increase in leptin, occurring around postnatal days 4-10 (Ahima, et al. 1998), the time when connections between hypothalamic neurons are developing. Nevertheless, despite this elevation in plasma leptin levels, neonates maintain a high level of food intake, and the responses to exogenous leptin administration are absent, until around the time of weaning. This is due to the fact that during this period leptin has no effect on appetite, but it seems to exert direct neurotropic actions, promoting neurite outgrowth and the establishment of hypothalamic circuitry, instead of regulating energy balance (Cottrell, et al. 2010).

Recent studies provide convincing evidence that leptin specifically promotes the development of axonal projections from ARC to PVN since day 4 of postnatal life, increasing inhibitory synapses and reducing excitatory synapses in the hypothalamus, and hence, affecting sensitivity to this hormone in adulthood [reviewed by (Keen-Rhinehart, et al. 2013)]. In this sense, a disruption in the postnatal leptin surge can alter the formation of neuronal pathways and can contribute to the later development of obesity (Martin-Gronert and Ozanne 2013). In fact, in leptin-deficient *ob/ob* mice, the aforementioned projection pathways regulating appetite are permanently disrupted causing reducing ARC axonal densities (Ahima, et al. 2000) and reducing AgRP and α -MSH fibre density in the PVN. This effect is reversed by chronic leptin injection during the first week of life (Bouret 2009).

Besides the role of leptin promoting the development of ARC neuronal projections, leptin is also required for normal neuronal and glial maturation in the mouse nervous system (Ahima, et al. 1999), adult neuronal hippocampal neurogenesis (Garza, et al. 2008) and dendrite formation (O'Malley, et al. 2007). In this sense, neonatal leptin surge has been related with neuron differentiation and migration, whereas low leptin levels have been associated with the maintenance of neural progenitor cells (Udagawa, et al. 2007).

Magnitude and timing of the leptin surge can be changed by maternal and neonatal nutritional environment during the perinatal period, such as maternal nutrient restriction (Martin-Gronert and Ozanne 2013). On the one hand, perinatal undernutrition, especially during fetal life, has been shown to result in a drastic reduction or even lack of plasma leptin surge in rats and increased hypothalamic ObRb protein expression at birth. This leads to permanent changes in hypothalamic ingestive behaviour circuits (Cottrell, et al. 2010; Delahaye, et al. 2008; Keen-Rhinehart, et al. 2013). Moreover, maternal isocaloric low-protein diet during gestation, which is associated with intrauterine growth retardation of their offspring, has also been described in rats to reduce and delay normal leptin surge in offspring from dams fed an isocaloric low-protein diet (Bautista, et al. 2008). On the other hand, subcutaneous injection of leptin (2.5 μ g/g body weight/day), from 5.5 to 10.5 days of age, has been shown to induce a premature leptin surge in intrauterine normal nourished mice. This unexpectedly led to accelerate weight gain of these animals under HF-diet feeding conditions (Yura, et al. 2005). Therefore, a premature leptin surge seems to be associated with an early-onset ARC leptin resistance, which can permanently alter central regulation of food intake, leading to accelerated HF-diet-induced obesity [reviewed by (Picó, et al. 2012)].

Leptin resistance

A majority of obese individuals are leptin resistant because they have increased levels of leptin, however, they do not respond to rising endogenous leptin levels, concerning its anorexigenic action via the ARC of the hypothalamus. Hence, leptin resistance is defined as the reduced ability of leptin to suppress appetite and weight gain (Ahima, et al. 2000; Zhou and Rui 2013). Therefore, leptin resistance has been considered as the main risk factor for the pathogenesis of overweight and obesity and may represent an integrated marker for the inextricably linked disease states of obesity, metabolic syndrome, insulin resistance, type II diabetes, hypertension, atherothrombosis and myocardial disease (Martin, et al. 2008)

Several mechanisms have been proposed to explain leptin resistance, among them, are of interest: genetic mutations, impairment in leptin transportation across the blood-brain barrier, reduction of leptin-mediated JAK2-STAT3 signalling, reduction of the levels of leptin receptor in the cell surface and induction of negative regulators such as SOCS3 (Martin, et al. 2008; Zhou and Rui 2013).

Concerning genetic mutations, a mutation in the *ob* gene producing leptin that is secreted but is ineffective at the signalling level, can lead to hyperleptinemia and leptin resistance. Similar results are observed if mutations in the gene encoding for the leptin receptor occur. In this sense, *Zucker* rats have dysfunctional leptin receptors, fact that causes hyperleptinemia and leptin resistance (Chen, et al. 1996). Such mutations in a single gene are uncommon in the typical obese population, although some cases have been found in humans. However, polygenetic inheritance patterns in other gene products, exerting influence on the leptin axis, are significantly contributing to the genetic predisposition to leptin resistance (Martin, et al. 2008). In this sense, deficiency of leptin, leptin receptor and STAT3 in POMC neurons induces hyperphagia and impairs thermogenesis leading to morbid obesity (Ahima, et al. 2000). In contrast, the loss of orexigenic peptides, attenuates obesity in leptin-deficient *ob/ob* mice (Ahima and Lazar 2008).

Most recently, the concept of “leptin resistance” has been challenged by an alternative concept, the “hypothalamic leptin insufficiency” (Rabe, et al. 2008). The major assumption of this postulation is that the blood-brain barrier restricts the leptin transport between the bloodstream and the brain neurons in response to hyperleptinemia, resulting in leptin insufficiency at multiple target sites in the brain (Rabe, et al. 2008)

Attenuation of leptin sensitivity in the brain leads to excess lipid accumulation in adipose tissue, muscle, liver and also in pancreas. This results in impaired insulin sensitivity and secretion (Rabe, et al. 2008). This lipid storage, as it occurs in obesity and the metabolic syndrome, is promoted because AMPK fails to inhibit ACC; this results in elevation of malonyl-CoA levels and in a simultaneous blockage of the FAO through inhibition of CPT1, leading to an increase in the synthesis of TGs and fatty acids [reviewed by (Aguilera, et al. 2008)]. Moreover, excess lipid accumulation also leads to formation of ceramide and various lipid metabolites that ultimately impair insulin sensitivity in liver and muscle, as well as insulin secretion (Ahima and Lazar 2008).

In addition, hyperleptinemia also can contribute to the development of hypertension with simultaneous resistance to the metabolic actions of leptin. In this sense, transgenic mice overexpressing leptin (whose elevated plasma leptin levels are comparable to those seen in obese subjects) exhibit elevated systolic blood pressure and urinary catecholamine excretion [reviewed by (Konner and Bruning 2012)].

2.2.2. Insulin

Insulin is a polypeptide of 51 amino acids produced by pancreatic β -cells in the islets of Langerhans. Insulin is secreted in response to an increase in glucose concentration and acts principally in peripheral organs regulating glucose homeostasis, lipid homeostasis, synthesis of proteins and gene expression (Niswender and Schwartz 2003). In addition to its peripheral functions, it has been more recently discovered that insulin, like leptin, is also involved in energy homeostasis, through their central action in the ARC [reviewed by (Pang and Han 2012)].

Insulin acts peripherally regulating glucose homeostasis by inhibiting endogenous glucose production and stimulating glucose uptake in liver, skeletal muscle and WAT. Concretely, insulin action on hepatocytes suppresses gluconeogenesis and glycogenolysis, and increases glycolysis and glycogen synthesis, resulting in decreased glucose output from the liver (Konner and Bruning 2012).

Insulin also regulates lipid homeostasis. Particularly, insulin is an antilipolytic agent and the main activator of energy storage in WAT, up-regulating lipogenesis (Cripps, et al. 2005). In this sense, mice with adipocyte-specific knockout of the *Insr* gene have a decrease in the ability of insulin to suppress lipolysis, as well as impaired insulin-stimulated glucose uptake and TG synthesis in adipocytes; therefore, these mice showed a reduction in fat mass and protection against obesity induced by a HF-diet or hypothalamic injury (Bluher, et al. 2002). Insulin effects on hepatic lipogenesis are predominantly mediated by SREBP1c, which in turn increases transcription of genes required for cholesterol, fatty acid, TG and phospholipid synthesis, such as *Acc* and *Fasn* (Rask-Madsen and Kahn 2012).

Furthermore, insulin also plays an important role in myeloid cells (monocytes, macrophages and neutrophils) regulating macrophage invasion in WAT and the development of obesity-associated insulin resistance. Therefore, deletion of the *InsR* in myeloid cells has shown to cause reduced accumulation of macrophages in WAT during HF feeding; as consequence, animals with deletions of the *Insr* gene have reduced circulating levels of TNF α , reduced activation of stress kinases in muscle, and are protected from the development of insulin resistance upon HF-diet feeding (Rask-Madsen and Kahn 2012).

In addition to its multiple peripheral functions, insulin, like leptin, also plays a pivotal role in the CNS, concretely, at hypothalamic level, regulating body weight, energy homeostasis, peripheral fat and glucose metabolism (Konner and Bruning 2012; Pang and Han 2012). Circulating insulin is transferred to the CNS using a receptor-mediated mechanism across the blood-brain barrier in proportion to its circulating levels (Baura, et al. 1993). Once in the CNS, insulin acts via its receptor (*InsR*) in the ARC, and regulates energy homeostasis by stimulating the expression of the anorexigenic neuropeptides (POMC and CART) and by inhibiting the expression of the orexigenic neuropeptides (NPY and AgRP) (Pang and Han 2012).

Insulin triggers its transduction pathway by activating its intrinsic tyrosine kinase activity (**Figure 2**), after binding its receptor, leading to tyrosine phosphorylation of the insulin receptor substrate (IRS) (Baskin, et al. 1998; Valassi, et al. 2008; Varela and Horvath 2012). Interestingly, insulin signalling pathway converges here with the leptin signalling pathway, the activation of PI3K (Havel 2004). Therefore, PI3K pathway is likely to mediate common actions of insulin and leptin as peripheral signals that converge on the CNS in the hypothalamic regulation of energy balance, metabolic homeostasis and feeding and neuroendocrine functions (Davis, et al. 2010). In addition, PI3K pathway of insulin signalling also results in AKT activation, which leads to prompt several cellular events, such as stimulation of glycogen

synthesis, inhibition of gluconeogenesis, and stimulation of glucose uptake (Konner and Bruning 2012).

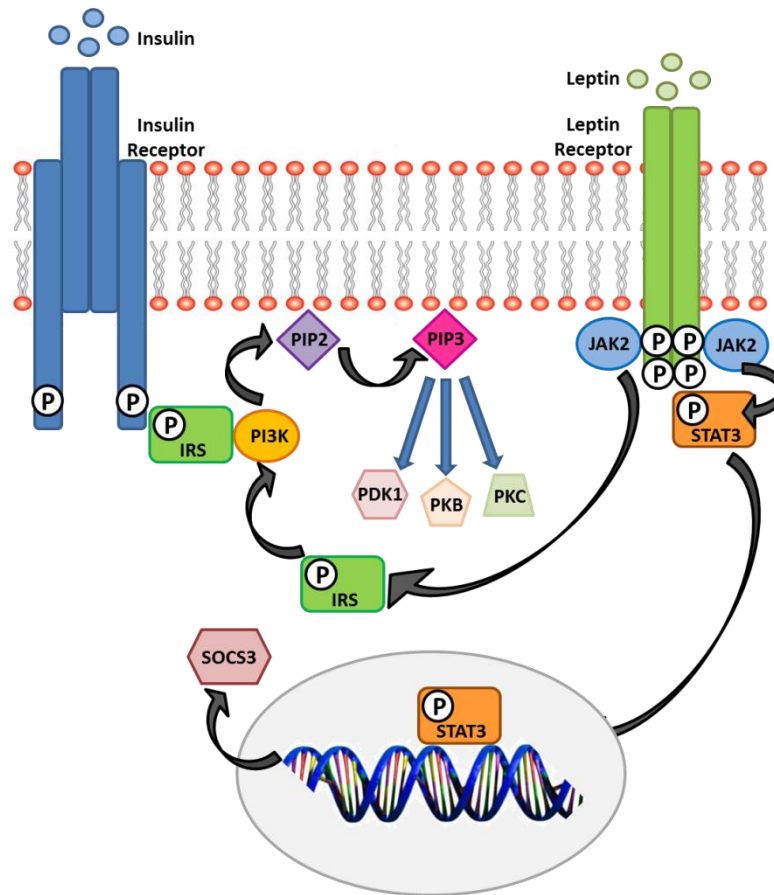


Figure 2. Schematic insulin and leptin intracellular signal transduction. Insulin receptor (InsR) is a disulfide-bonded dimer with two α and β subunits. Upon insulin binding to the extracellular portion of α subunits, a tyrosine kinase intrinsic to the intracellular β subunit is activated leading to phosphorylation of multiple tyrosine residues on β subunits. Phosphorylation of these residues creates a binding site for insulin receptor substrate (IRS) proteins. Then, IRS proteins bind to and activate the enzyme phosphatidylinositol 3-kinase (PI3K) which phosphorylates phosphatidylinositol (4,5) bisphosphate (PIP2) to phosphatidylinositol (3,4,5) triphosphate (PIP3). PIP3 in turn recruits and activates numerous other downstream molecules including phosphoinositide dependent kinase 1 (PDK1), protein kinase B (PKB/AKT), and protein kinase C (PKC). The leptin receptor (ObRb) belongs to the cytokine receptor family and consists of a single membrane spanning domain. When leptin binds to its receptor, it induces activation of Janus Kinase 2 (JAK2) by autophosphorylation of tyrosine residues on JAK2, allowing receptor dimerization. Then, JAK2 phosphorylates residues of Tyr⁹⁸⁵, Tyr¹⁰⁷⁷, and Tyr¹¹³⁸ on the intracellular portion of the ObRb. Signal transducer and activator of transcription 3 (STAT3) molecules are then recruited via phosphotyrosine binding, and are, in turn, activated by tyrosine phosphorylation mediated by JAK2. Activated STAT3 dimerizes, translocates to the nucleus, and activates the transcription of target genes. Suppressor of cytokine signalling 3 (SOCS3) molecules are synthesized in response to STAT3 activation and terminates leptin signalling. Leptin also activates PI3K signalling via JAK2 mediated phosphorylation of IRS proteins. Adapted from (Niswender and Schwartz 2003).

Insulin resistance

Insulin resistance is a term that describes the inability of the pancreatic hormone insulin to maintain glucose homeostasis. Insulin resistance has been linked to obesity and other several clinical conditions such as hypertension, increased adiposity in the visceral region,

dyslipidemia, atherosclerosis, cardiovascular disease, polycystic ovaries syndrome and cancer [reviewed by (Konner and Bruning 2012)].

The link between obesity and insulin resistance is well recognized (**Figure 3**). A positive energy balance leads to increased storage of fat in adipose tissue; however, with the development of obesity the ability of adipocytes to store TGs is impaired or exceeded. As consequence, fat is stored in other cell types, including liver and skeletal muscle. Ectopic lipid and their metabolites or increased concentrations of circulating free fatty acids cause insulin resistance in muscle and other peripheral tissues (Rask-Madsen and Kahn 2012). Moreover, WAT is infiltrated with mononuclear cells and other immune cells because adipocytes express monocyte chemoattractant protein-1 and other cytokines. This infiltration can also be produced in response to adipose tissue hypoxia or adipocyte death. Consequently, it is established a chronic inflammatory state and hence, proinflammatory cytokines, such as TNF α and IL-6, are released promoting insulin resistance in skeletal muscle, liver and other tissues (Rask-Madsen and Kahn 2012; Sell, et al. 2006).

Resistance to the effects of insulin represents a key process in the development of type II diabetes mellitus. In the prediabetic state, pancreatic β -cells can increase insulin secretion in order to compensate for insulin resistance and to maintain glucose homeostasis (compensatory hyperinsulinemia). Nevertheless, if this condition persists, β -cell dysfunction occurs and insulin secretion is suppressed, hence, glucose homeostasis gets impaired and type II diabetes mellitus becomes established (Konner and Bruning 2012).

In skeletal muscle, insulin resistance decreases receptor binding and phosphorylation of the insulin receptor and IRS1. There is also a dramatic reduction of insulin signalling through PI3K and AKT, fact that ultimately results in a decrease in translocation of the glucose transporter GLUT4 to the plasma membrane and impaired insulin-stimulate glucose transport into the cell (Konner and Bruning 2012; Rask-Madsen and Kahn 2012).

In liver, insulin fails to adequately suppress hepatic glucose production, fact that represents a hallmark of progression from insulin resistance to overt diabetes mellitus (Rask-Madsen and Kahn 2012). At the molecular level this is represented by an increased expression of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose 6-phosphatase and a decreased expression of enzymes that regulate glycogen synthesis and glycolysis, including glucokinase and pyruvate kinase (Michael, et al. 2000). Although the insulin suppression of glucose production is completely lost in liver due to insulin resistance, insulin continues promoting lipid synthesis increasing circulating levels of free fatty acids and TG, fact that leads to hypertriglyceridemia. In addition, hepatic insulin resistance also contributes to hyperlipidemia by downregulation of the LDL receptor. Therefore, it is well recognized that hepatic insulin resistance contributes to the classical hyperglycemia, hyperinsulinemia and hypertriglyceridemia observed in humans with type II diabetes mellitus (Konner and Bruning 2012).

In the brain, impaired insulin signalling contributes to obesity by failing to suppress appetite. Moreover, brain insulin resistance also leads to glucose intolerance by impairing the ability of insulin to suppress hepatic glucose output through innervation of the liver (Rask-Madsen and Kahn 2012).

Furthermore, clinical studies indicate that insulin resistance at the vascular wall could be important in the development of cardiovascular pathologies in insulin-resistant and diabetic patients due to the acceleration of atherosclerosis (Konner and Bruning 2012). Normally, insulin acts on vascular endothelial cells affecting endothelial function beyond regulating blood

flow or capillary recruitment; hence, insulin activates both antiatherosclerotic and proatherosclerotic mechanisms in vascular endothelial cells. It has been suggested that the lack of insulin actions mediated by the activation of IRS1/PI3K/AKT pathways, in vascular endothelial cell, could be the main cause for the development and progression of atherogenesis and microvascular disease in type II diabetes mellitus patients. In addition, insulin resistance in smooth muscle cells may also contribute to the development of diabetes-associated cardiovascular disease (Konner and Bruning 2012; Rask-Madsen and Kahn 2012)

In the context of insulin resistance, adiponectin is a hormone of particular interest. Concretely, adiponectin is produced exclusively by the adipose tissue and its plasma levels correlate negatively with BMI, insulin and TG levels, and positively with high-density lipoprotein (HDL) cholesterol, in obese adults (Fliers, et al. 2003). Adiponectin has multiple beneficial effects on a cluster of obesity-related metabolic and cardiovascular dysfunctions. Like leptin, adiponectin displays antisteatotic activity in non-adipose tissues, but the most important role of adiponectin is its ability to improve insulin sensitivity through stimulation of the insulin receptor tyrosine kinase activity (Wang, et al. 2009).

Two adiponectin receptors have been identified: adipoR1 (abundantly expressed in skeletal muscle) and adipoR2 (present predominantly in the liver) (Wang, et al. 2009). Adiponectin contributes to enhance insulin sensitivity through binding its receptors, adipoR1 and adipoR2, prompting the phosphorylation and activation of AMPK. Activation of AMPK in its major target tissues, including skeletal muscle, liver, heart, endothelium, adipocytes and brain, leads to stimulate glucose utilization and FAO, and hence, to enhance whole-body insulin sensitivity (Rabe, et al. 2008). Concretely, AMPK attenuates by phosphorylation ACC activity, which results in a reduction of lipid synthesis and an enhancing of FAO by blocking the production of malonyl-CoA. In addition, activation of AMPK downregulates the expression of *Srebp1c*, which in turn leads to downregulate the expression of genes involved in lipogenesis, including *Acc*, *Fasn*, and glycerol-3-phosphate acyltransferase (*Gpat*) [reviewed by (Wang, et al. 2009)]. Therefore, considering this upregulation of adiponectin/adiponectin receptors, enhancing adiponectin receptor function may represent a potential strategy to treat obesity-linked insulin resistance (Rabe, et al. 2008).

Additionally, adiponectin has been also described to have anti-atherogenic and anti-inflammatory properties (Wang, et al. 2009).

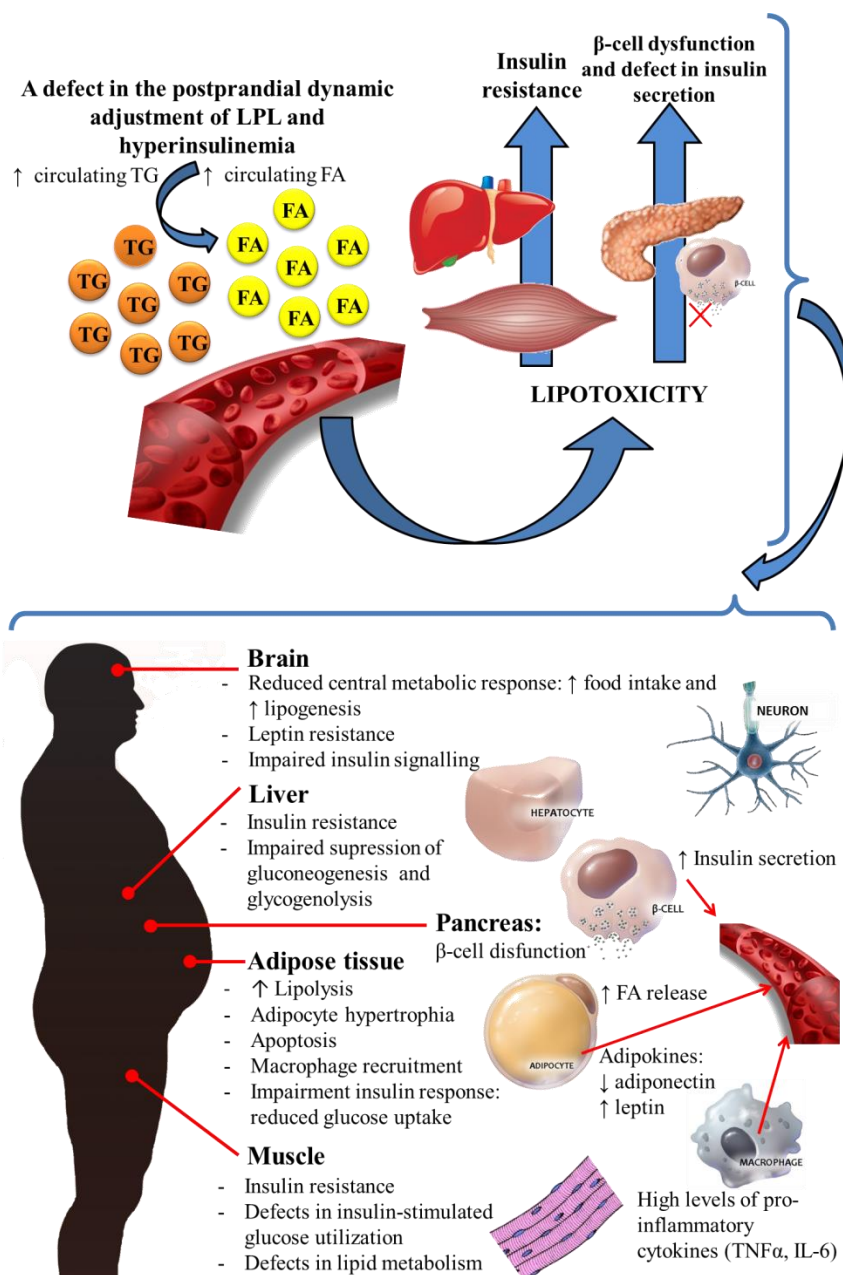


Figure 3. Overview of the pathophysiology of obesity-related insulin resistance. Obesity is characterized by a chronic positive energy balance which leads to an increased storage of fat in adipose tissue. With the development of obesity, the ability of adipocytes to store triglycerides (TG) is impaired or exceeded. Consequently, fat is stored in other ectopic tissues, including liver and skeletal muscle. Moreover, circulating TG and free fatty acid (FA) levels are also increased in obesity due to a defect in the postprandial dynamic adjustment of lipoprotein lipase (LPL) and due to a postprandial impairment of the normal ability of adipose tissue to buffer FA excess. Under these conditions, all tissues are chronically exposed to very high concentrations of FAs and TGs; this situation is known as “lipotoxicity”. Lipotoxicity gradually impairs insulin action on the liver and muscle, aggravating insulin resistance. As long as pancreatic β-cells can adapt to insulin resistance in peripheral tissues with an appropriate increase in insulin secretion, normal glucose homeostasis can be maintained. However, lipotoxicity on pancreas can also produce β-cell dysfunction, which results in a defect in insulin secretion. Concurrently, fat tissue is infiltrated with macrophages and other immune cells. Adipose macrophages release pro-inflammatory cytokines, such as TNFα and IL-6 that also contribute to insulin resistance in muscle, liver and other tissues. In the brain, impaired insulin signaling and compromised nutrient sensing contributes to obesity by failing to suppress appetite. Brain insulin resistance also leads to glucose intolerance by impairing the ability of insulin to suppress hepatic glucose output through innervation of the liver Adapted from (Rask-Madsen and Kahn 2012).

2.3. Calorie restriction

Calorie restriction is a dietary intervention defined as a reduction in macronutrient intake, below the amount of calories that would be consumed *ad libitum*, with maintenance of a sufficient micronutrient intake (Minor, et al. 2010). In general, calorie restriction is set at around $\geq 10\%$ reduction of energy consumption in human studies and 20% or higher in rodent species (Bales and Kraus 2013).

The balance between energy (calorie) consumption and energy expenditure is critical for the quality of life across the lifespan. A clear example of this observation is the fact that an increased calorie intake together with a sedentary lifestyle is responsible for the epidemic of obesity and for the deterioration of the health span because obesity, in turn, also increases the risk for chronic inflammatory disorders such as hypertension, type II diabetes, coronary heart disease and other related metabolic alterations (Gonzalez, et al. 2012). However, in contrast to the detrimental effects of eating an excess of energy, a restriction of calorie intake below the amount required for weight maintenance appears to have a wide range of benefits. Particularly, calorie restriction has shown to slow the aging process, extend lifespan and improve the health span (Bales and Kraus 2013).

Mammalian experimental studies and also preliminary studies on humans have shown that calorie restriction, in addition to its commonly accepted effect on increasing lifespan, also benefits other processes that contribute to overall health. In this regard, calorie restriction has been considered the most efficient way to promote weight loss in the population. Moreover, moderate calorie restriction has been shown to prevent or reverse the damaging effects of type II diabetes, hypertension, cardiovascular disease, chronic inflammation and other age-associated metabolic diseases, such as blood glucose regulation [reviewed by (Gonzalez, et al. 2012)].

Another benefit of calorie restriction is the prevention of free radical formation. Concretely, long-term calorie restriction reduces the age-associated accumulation of oxidative damage to proteins, lipids and DNA (Sohal and Weindruch 1996). This reduction can be due to several processes, including a decrease in the generation of reactive oxygen molecules, an increased efficiency of protective processes, an increase in repair activity, or a combination of these processes. In this sense, calorie restriction has been shown to enhance autophagy and DNA repair systems, and up-regulates endogenous enzymatic and non-enzymatic antioxidative defense mechanisms (Omodei and Fontana 2011).

The biological mechanisms by which calorie restriction decreases insulin resistance and improves glucose metabolism are still not fully elucidated. However, it has been proposed that calorie restriction may reduce plasma levels of pro-inflammatory cytokines, peptides and other factors produced normally by the adipose tissue (Gonzalez, et al. 2012).

At gene expression level, calorie restriction may induce the activity of genes that protect cells from the damaging action of harmful agents. In this sense, it has been shown that calorie restriction increases the induction of the gene encoding one of the hepatic scavenging proteins (*Hsps70*) in response to heat stress (Heydari, et al. 1993). Moreover, several observations suggest that a low-calorie diet also has an impact on the expression of inflammatory genes in peripheral mononuclear cells (PBMCs) of obese patients (Crujeiras, et al. 2008).

All in all, it has been assumed that calorie restriction is a low intensity stressor that provokes a survival response in the organism, helping it to tolerate adversity by activating longevity pathways (Omodei and Fontana 2011). Moreover, the antioxidant and anti-inflammatory

properties of calorie restriction make this nutritional intervention approach as an interesting and promising alternative to prevent and control cardiovascular disease (Gonzalez, et al. 2012).

Despite the fact that calorie restriction is the only intervention known to date that consistently decreases the biological rate of aging and increases both average and maximal lifespan, some detrimental effects have also been observed. In this sense, studies with rodents suggest that 40-60% calorie restriction has detrimental effects on the innate immune response with impaired ability to control infections by monocytes/macrophages (Jolly 2004). Another worrisome concern about calorie restriction, especially in humans, is the potential development of symptoms of eating disorders. It was observed that calorie restriction or the intent to restrict intake has been associated with the onset of eating disorders, such as anorexia, bulimia nervosa and binge eating disorder. Hence, there is a need to examine both the benefits and potential harms of calorie restriction in humans [reviewed by (Redman and Ravussin 2011)].

Another important fact to take into account is the moment of life in which the calorie restriction is imposed, because it is already accepted that certain stimuli experienced during critical periods of development, such as pregnancy and lactation, may cause permanent alterations in the development and organization of tissues and organs that can lead to irreversible changes in the structure and function of the body, programming the metabolic health of an individual in adult life (Martin-Gronert and Ozanne 2013; Picó, et al. 2012). Further information about the effects of the exposure of calorie restriction either during pregnancy or lactation will be given in the next section (section 3).

3. Developmental origins of obesity: importance of early life nutrition

There is a growing body of evidence, from epidemiological studies in humans and animal models, showing that early life environmental factors, especially early nutrition during prenatal and neonatal periods, have profound effects on genome regulation modifying, in turn, the susceptibility of an individual to develop a wide spectrum of metabolic diseases later in life (Guilloteau, et al. 2009; Martin-Gronert and Ozanne 2013). In this sense, it has been estimated that 62% of the variation in human birth weight results from the intrauterine environment, including maternal gestational nutrition, compared with 20% and 18% resulting from maternal and paternal genetic heredity, respectively (Holt 2002).

The idea that nutritional and environmental factors during early life have lasting consequences on the future metabolic health of an individual was firstly developed in 1990, by Barker, with the hypothesis of the “foetal origin of adult diseases” (Barker 1990). For most organs and systems, the critical period of plasticity is during intrauterine development (Barker 2007). Based on the observation that environmental changes during a critical period of life can readjust the developmental path, the concepts of plasticity and programming appear. Developmental plasticity is defined as the ability of an organism to change his phenotype in response to changes in the environment (Cota and Allen 2010). If this change or adaptation is permanent, it is considered a “programming”. Programming is defined as the induction, silencing or restriction of development of a permanent somatic structure or physiological system—caused by stimuli, nutritional or disturbing factors acting during a sensitive time period—with long term effects on foetal growth quality and on the metabolic responses of the offspring (McMillen and Robinson 2005). Interestingly, although developmental programming is induced by processes of developmental plasticity, once induced, the organism has a reduced capacity to reverse the developmental trajectory chosen, making developmental programming irreversible in a particular nutritional range (Bateson, et al. 2004).

Pregnancy and lactation are revealed as these critical periods where maternal energy status, diet, body composition and health have long-term effects on metabolic mechanism in the offspring (Sullivan and Grove 2010). Barker and collaborators observed that maternal undernutrition during pregnancy leads to metabolic adaptations in the foetus to increase its survival by producing an individual who can maximize the storage of calories during times of plenty and minimize loss of energy stores during times of famine. Thus, Hales and Barker proposed that poor foetal and early postnatal nutrition imposes mechanisms of nutritional thrift upon the growing individual; it is their hypothesis of the “Thrifty phenotype” (Hales and Barker 2001). In the past, such evolutionary changes were beneficial. However, nowadays and in developed countries, where there is an overabundance of highly palatable food, which can be obtained easily, with minimal energy expenditure, such individuals show a rapid growth throughout childhood, adolescence and adulthood, a great increase in adiposity and hence, a great risk of becoming obese and developing the metabolic disorders associated to obesity, such as insulin resistance, in adulthood (Martin-Gronert and Ozanne 2013).

Therefore, bearing in mind that nutrition during early life can program body composition and the metabolic health of an individual in adult life, Martorell *et al.* (Martorell, et al. 2001) proposed the effects of nutrition in early life throughout three distinct hypotheses: 1) overnutrition increases the risk of later fatness; 2) the opposite, i.e. undernutrition, is also associated with increased risk of fatness; and 3) optimal nutrition during infancy, represented by breastfeeding, is protective against future obesity.

3.1. Programming effects of maternal calorie restriction during gestation

Initial programming studies have mainly focused on the effects of intrauterine malnutrition. Epidemiological studies in humans suggest that poor early nutrition has different effects on the metabolic health of the offspring depending on when in gestation occurs (Ravelli, et al. 1976). Poor nutrition of the mother during maximal placenta growth has been reported to influence foetal growth and body weight in human babies. Concretely, food restriction during early gestation influences the cardiovascular system, reflected as an increased risk of coronary heart disease in humans. Whereas, nutrient restriction during late gestation, coincident with the period of maximal foetal growth, affects intermediary metabolism, particularly, glucose and insulin homeostasis, leading to an increased risk of type 2 diabetes [reviewed by (Guilloteau, et al. 2009)].

The association between intrauterine malnutrition and subsequent development of obesity has been supported by the emblematic example of the Dutch famine that devastated the West part of Holland during the last 6 months of World War II (Ravelli, et al. 1976). In October 1944, the German authorities blocked all food supplies to the occupied west of The Netherlands. These were restored immediately after liberation on May 1945. Therefore, children exposed to famine *in utero* during the known as the “hunger winter” were well nourished in childhood and, in consequence, had accelerated weight gain (Lumey, et al. 2007). The hunger winter cohort was used to examine how maternal undernutrition during specific gestational time windows affects the subsequent life course of offspring who experienced the famine *in utero*. The results of these studies showed that 19-year-old men, exposed to the acute famine *in utero* during the first half of gestation, displayed increased obesity rates. However, exposure to famine during the last trimester of gestation and in early postnatal life was associated with reduced obesity (Ravelli, et al. 1976). In addition to the studies of the Dutch hunger winter, many other epidemiological studies have indicated that obesity and impaired glucose tolerance are highly prevalent in subjects born from dams that were exposed to famine during gestation. Another

example is the Great Chinese famine that affected the Chongqing population during 1959-1961, which was more devastating in rural areas and caused millions of deaths. Individuals exposed *in utero* to the Chinese famine were at increased risk of overweight and hyperglycemia in adult life (Li, et al. 2010).

From human studies it is virtually impossible to identify the underlying mechanisms involved in developmental programming. For this reason, animal models have become a mainstay of research and have supported the observations made in epidemiological studies. Most of the studies associating foetal malnutrition and adult obesity are from rat models. Indeed, different outcomes have been described regarding subsequent offspring body weight depending on the type, severity and timing of the restriction, and also on the gender of the progeny (Picó, et al. 2012; Symonds, et al. 2004). In these sense, 50% maternal calorie restriction during the first two weeks of gestation led to obesity development in male, but not in female, offspring shortly after weaning (Jones and Friedman 1982). In addition, Desai and colleagues observed that the offspring of rat dams exposed to 50% calorie restriction from day 10 of gestation until delivery displayed a significant intrauterine-growth restriction; however, these pups experienced a markedly increased weigh gain after birth, even bigger than body weight of control pups, under *ad libitum* feeding conditions. Interestingly, male offspring also had significantly higher body weights than females from the age of 4 weeks, and this difference became perceptibly accentuated with advancing age (Desai, et al. 2005). Other studies consisting in less severe maternal calorie restriction were also performed. In this regard, Vickers and colleagues performed a moderate 30% maternal calorie restriction throughout pregnancy, which resulted in male offspring developing hyperphagia and greater accumulation of adipose tissue without any change in body weight (Vickers, et al. 2000).

Many animal studies coupled with new experimental tools have showed that the mechanisms, by which nutrition during foetal development lead to influence the propensity to develop obesity and other related metabolic alterations later in life, are very complex and may include epigenetic modifications and also changes on key structures, such as the hypothalamus and the peripheral nervous system [reviewed by (Picó, et al. 2012)]. In this regard, nutrition during critical windows of foetal development, especially the availability of dietary methyl donors and cofactors in food, may induce DNA methylation patterns (epigenetic modification generally associated with transcriptional repression) across a number of tissues and organs, leading to premature changes and conferring an enhanced susceptibility to adult diseases in later life (Vickers and Sloboda 2012). Interestingly, in rodents, the hypothalamic *Pomc* gene promoter region is a key target of epigenetic changes, following perinatal nutritional manipulation. Concretely, the *Pomc* promoter was found to be less methylated in weanling rat offspring from low-protein isocaloric fed dams (Coupe, et al. 2010), whereas hypermethylation of the hypothalamic *Pomc* gene promoter and also of insulin receptor promoter were reported in weanling pups raised in small litters, which in turn could contribute to hypothalamic leptin/insulin resistance and obese phenotype observed in this animal model (Plagemann, et al. 2009).

Furthermore, early nutrition, including maternal nutrient restriction or maternal HF-diet feeding during perinatal period, has been shown to modify the structure and physiology of the hypothalamus (Remmers and Deleamarre-van de Waal 2011). In particular, 50% calorie restriction during pregnancy and lactation results in an increase in cell proliferation in PVN, VMN and ARC hypothalamic areas of pups (Coupe, et al. 2009), a reduction of nerve fibres projection from the ARC neurons to the PVN, and a diminished expression of *Pomc* in neonate rats (Delahaye, et al. 2008). García *et al.* also showed that a less severe maternal calorie restriction (20%) during the first 12 days of pregnancy also altered hypothalamic structure in

weaned rats, by decreasing the presence of total number of cells, particularly NPY-neurons (García, et al. 2010). Interestingly, these changes occurring in hypothalamic structures are also accompanied, in most cases, by alterations in the expression of neuropeptides. In these regard, García *et al.* also showed that the offspring of calorie restricted dams during pregnancy showed reduced hypothalamic mRNA expression levels of *Pomc* and *Npy* (only female pups) and of *Obrb* and *Insr* (both male and female pups) at weaning (García, et al. 2010).

The identification of such mechanisms has made possible the development of strategies addressed to revert or prevent at early ages the metabolic programming for obesity and its related metabolic disorders. In this sense, Konieczna and coworkers (Konieczna, et al. 2013) showed that oral supplementation with physiological doses of leptin during lactation is able to revert, at least in part, most of the detrimental effects in the offspring on hypothalamic structure and function caused by maternal calorie restriction during gestation. Therefore, developmental malprogramming during foetal life can be reversed by nutritional interventions during the neonatal period, such as the intake of appropriate doses of leptin through lactation, which in turn, could be considered a strategy to revert or prevent programmed obesity and its metabolic-related alterations (Konieczna, et al. 2013). Identification of other strategies able to revert or ameliorate detrimental programmed effects due to adverse conditions during gestation is of great interest.

3.2. Programming effects of maternal calorie restriction during lactation

The early postnatal period is also critical for determination of long-term body weight. Unlike the known negative effects of calorie restriction during foetal life, increasing the risk of developing obesity, the lasting consequences of maternal undernutrition during the suckling period are uncertain. In fact, studies in humans are scarce mainly due to ethical aspects; however, some interventional studies in humans have been performed. McCrory and coworkers evaluated whether maternal weight loss by 35% calorie restriction, with or without aerobic exercise, had adverse effects on lactation. They showed that short-term weight loss of 1 Kg per week, by a combination of dieting and aerobic exercise for 11 days, appears safe for breastfeeding mothers and no significant changes were detected neither in the amount and energy output of milk nor in frequency of nursing and weight gain of infants (McCrory, et al. 1999). In another study, overweight lactating women, who were exclusively breastfeeding, were subjected to calorie restriction of 500 Kcal less than control mothers, and also to moderate exercise between 4 and 14 weeks postpartum. These lactating women also showed a moderate weight loss of 0.5 Kg per week and no alterations on normal weight gain of infants were observed (Lovelady, et al. 2000). Further studies in humans are needed to assess whether this maternal nutritional condition during lactation has long-term effects on their offspring, because all the studies performed until date have been only focused on the short-term effects.

Several rodent models have also been used to study the effects of nutrition during the suckling period. It is known that the quantity of food available during suckling is also a determinant issue for the development of obesity in pups (Levin 2006). In these sense, one approach to alter the intake of neonates consists in the reduction or the increase in litter size. Small litter size leads to a relative excess of milk for each pup and changes the composition of maternal milk, leading to increased fat content. It results in hyperphagia and increased growth rates leading to permanent overweight and increased fat deposition (Plagemann, et al. 1999b). However, an increased litter size from 10 to 20 pups during lactation resulted in lower body weight and food intake of both male and female restricted pups in adulthood. These animals displayed reduced body dimensions at weaning and also showed incomplete catch-up growth size afterwards

(Remmers, et al. 2008a). Velkoska and collaborators also demonstrated that pups raised in large litters (18 pups per litter) were lighter than their controls and remained significantly lighter throughout the study, however no evidence of incomplete catch-up growth was observed (Velkoska, et al. 2008). Other approaches involve maternal calorie restriction through the lactating period. This condition also leads to lower body weight of pups at weaning; however a severe nutritional deprivation (67%) of lactating dams caused a considerable retardation of the rate of growth in pups, probably because dams were unable to deliver sufficient amount of milk to sustain the normal growth (Boxwell, et al. 1995). Vicente and coworkers also showed that 65% maternal protein restriction or maternal energy restriction (to the same amount of calories eaten by the protein restricted group) during lactation in rat dams resulted in lower body weight of offspring at weaning. In adulthood, animals whose mothers were fed the protein restricted diet weighed significantly less than the controls. However, the animals whose dams were fed the energy restricted diet weighed more than the controls (Vicente, et al. 2004). Another study showed that female offspring of 50% calorie-restricted dams during the suckling period, although presenting a lower body weight than their controls, displayed increased food intake (Šefčíková and Mozeš 2002).

Therefore, animal studies show that maternal dietary restriction during lactation (total calorie or protein content) or by increase in the litter size during lactation results in a different programming of body weight and food intake control in the offspring, which may affect the propensity to develop obesity. It is shown that severe maternal deprivation during the suckling period leads to detrimental outcomes in the offspring (Boxwell, et al. 1995; Remmers, et al. 2008a); however, the effects on their offspring of a less severe maternal dietary restriction have not been directly explored.

Breast milk

Bearing in mind that nutrition during early life has a considerable influence on long-term body weight and future metabolic health, and considering that in animals and fully breastfed infants maternal milk is the only source of food during this period, it has been suggested that composition of maternal milk may have an important role in developmental programming (von Kries, et al. 1999). Besides the basal nutritive function of maternal milk, to promote the properly postnatal growth and provide all the nutritional needs of infants (Ip, et al. 2007), the mother is able to communicate metabolic information or even social cues to the infant directly through her milk, helping the infant to adapt to the variable nutritional and environmental conditions (Newburg, et al. 2010).

Breastfeeding has been reported to bring more advantages for the metabolic health of the infants than formula feeding. Concretely, breastfeeding has been associated with reduction of incidences and severity of infections in infants, prevention of allergies, prevention of some gastrointestinal pathologies and sudden infant death syndrome, as well as enhancement of cognitive development (Leung and Sauve 2005). Moreover, there is a growing body of evidence, from epidemiological studies, suggesting that breastfeeding allows appropriate metabolic programming by lowering postnatal growth rate and fat deposition during the first year of life, and hence, reducing the risk of obesity and its related metabolic disorders in childhood and adolescence (Gundersen 2008; Ip, et al. 2007; von Kries, et al. 1999).

Despite the beneficial effects of breastfeeding for the future health of the individual, changes in the composition of breast milk may have profound effects on its role in developmental programming. In this sense, maternal behavioural, maternal lifestyle or maternal nutrition during lactation may affect specific components contained in milk, either by changing the

composition of maternal milk or changing the integrity of milk components. These changes in breast milk composition may affect the metabolic programming of their offspring, modifying their propensity to develop obesity and other related metabolic alterations (Pico, et al. 2011).

Therefore, identification of these changes in breast milk composition—changes that underlie the better or worse metabolic health programming due to a particular nutritional environment of their dams during lactation—becomes of special interest nowadays.

In this sense, in order to identify changes in maternal milk composition, the identification of free amino acids and free glycation, oxidation or nitration adducts, which have been formed as a result of the degradation of misfolded proteins and are considered to be potential biomarkers of protein damage (Karachalias, et al. 2010; Thornalley 2008; Thornalley, et al. 2003) is of particular interest. Therefore, identification of these biomarkers of protein damage in breast milk samples from mothers exposed to a particular nutritional environment during lactation could help us to explain the way by which changes in the nutritional environments of lactating dams can affect the future metabolic health of their offspring.

4. Biomarkers of metabolic health

Searching for new biomarkers in the field of human health is an interesting way to obtain biological tools which can help us to improve health outcomes and reduce total health care costs in the long term. In particular, application of novel health biomarkers may improve the understanding of the mechanisms of disease, may provide new knowledge for the identification of processes to improve health management through the earlier diagnosis of disease and may help the delivery of more efficacious and safer therapies to modulate disease progression (OECD 2011).

Biomarkers in the field of human health are considered as measurable indicators of biological states, which can provide us information on the presence of disease, or susceptibility to disease, or predict patient response to therapeutic interventions (OECD 2011). From a clinical standpoint, the most useful biomarkers provide a definitive link to a very specific disease risk or condition. Additionally, nowadays, a huge effort is directed towards the identification of markers that provide the earliest possible indication of disease (Elliott, et al. 2007). Nevertheless, all these biomarkers allow identifying and quantifying disease or damage. This has led to major problems in demonstrating health benefits of food and establishing health claims. For this reason, the BIOCLAIMS project—“BIOmarkers of Robustness of Metabolic Homeostasis for Nutrigenomics-derived Health CLAIMS Made on Food” (BIOCLAMS, Grant agreement no. 244995) — attempts to identify new early healthy biomarkers through quantification of the robustness of the homeostatic mechanisms involved in maintaining optimal health, based on the assumption that the ability to maintain homeostasis in a continuously challenged environment is crucial for healthy ageing. This flags the way for the development of a new generation of robust biomarkers and their potentially related health claims made on foods.

A biomarker of health, to be optimal, must be analytically robust, sensitive, quantitative, practical and mechanism-linked so that implications of changes can be understood. Moreover, an optimal health biomarker should be based around measurements performed on accessible substrates, such as biological fluids (Elliott, et al. 2007). The major bottleneck in identification of biomarkers of health in humans, for predicting later metabolic alterations or for assessing their metabolic status, is the limited accessibility of tissues of healthy volunteers. In ill subjects that need to undergo surgery biopsies, both pathological and more normal surrounding tissue

samples can be easily obtained, whereas in patients without necessity of surgery and especially in healthy volunteers, collection of samples from internal organs such as liver, pancreas and visceral adipose tissue are not assessable for invasive investigations because of ethical reasons (Afman and Muller 2012). Therefore, less invasive sources for biomarkers identification are needed. Some of these new sources are PBMCs, a subset of white blood cells, which have emerged as a convenient surrogate tissue for biomarker identification in toxicological, nutritional and clinical studies (Rockett, et al. 2004).

Another important fact to take into account is that individual markers, used in isolation, are not able to measure health reliably. Therefore, in spite of individual markers, integrated multi-component biomarkers are required, because they would examine a far broader concept of health than simply individual parameters. According to this, the BIOCLAIMS project, in order to identify and validate multi-component markers of health, makes use of advanced analytical methodologies, including OMIC technologies—that enable to analyse the expression of large number of mRNA (transcriptomics), metabolites (metabolomics) or proteins (proteomics) — and other tools, such as the analysis of protein damage.

Particularly, transcriptomics techniques, such as whole genome microarray analysis, enable the unbiased measurement of total genome-wide effects on the cellular level and generate a comprehensive overview on molecular changes at gene expression level, to specifically interventions, such as nutritional interventions, in a given tissue at a given time; it is suitable for a first screening in regulatory networks (Kussmann, et al. 2006). Concerning metabolomics, it is a diagnostic tool that study the changes in metabolites (from body fluids, such as urine, blood and saliva) and whose goal is to isolate and characterize these metabolites in order to allow knowing the arrangements caused by a particular diet and how these changes may affect the metabolic health of an individual (Sales, et al. 2014). Proteomics is the science that studies the complete set of proteins involved in the biological processes and is suitable for discovering bioactive food proteins and peptides (Kussmann, et al. 2006; Sales, et al. 2014). Regarding protein damage, in physiological systems proteins undergo modifications (spontaneously, during aging or due to particular diseases) leading to change their structures and functions. As consequence, damaged proteins undergo proteolysis and free amino acids and free adducts are released and can be considered as robust biomarkers of protein damage (Karachalias, et al. 2010; Thornalley 2008; Thornalley, et al. 2003).

In this Thesis, two different tools have been used to identify biomarkers of metabolic health: 1) gene expression analysis using transcriptomic techniques; 2) analysis of protein damage using the technique of liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

4.1. Gene expression analysis in PBMCs

PBMCs are a subset of white blood cells from innate and adaptive immune systems, including lymphocytes and monocytes/macrophages, which travel through the entire body and respond to various internal and external signals.

Researches have focused on PBMCs as a potential source of biomarkers, because, contrary to adipose tissue, liver and muscle (tissues in which sample collection requires invasive biopsies), PBMCs are easily obtainable from healthy human volunteers. PBMCs can be easily isolated from blood, through venipuncture and can also be repeatedly collected in sufficient quantities in contrast to other tissues (de Mello, et al. 2012). PBMCs circulate throughout the body and can infiltrate the endothelium; hence, PBMCs are exposed to both exogenous and endogenous

stimuli from the adipose tissue and other metabolic tissues, as well as to all physiological changes, including changes in nutrient levels, and metabolism-related hormones (O'Grada, et al. 2014). Therefore, PBMCs may reflect systematic health (Afman, et al. 2014).

PBMCs have been shown to express about 80% of the genes encoded by the human genome (Liew, et al. 2006). Recent studies have proven that gene expression profile of PBMCs is a highly robust tool to distinguish a disease state from the healthy state and also to differentiate several phases/diagnostics of disease (Afman and Muller 2012; Bouwens, et al. 2008; Burczynski and Dorner 2006). In particular, gene expression profile of PBMCs could reflect the presence and the extent of different physio-pathological conditions of a person, such as coronary artery stenosis, chronic heart failure, hypertension, atherosclerosis, and inflammatory autoimmune diseases such as systemic lupus erythematosus [reviewed by (Afman, et al. 2014)]. Moreover, gene expression levels of PBMCs can reflect global gene expression profile of skeletal muscle (Rudkowska, et al. 2011) and can also reflect metabolic and immune responses of adipocytes or hepatocytes (Fuchs, et al. 2007). Furthermore, PBMCs can also reflect the responses to dietary interventions (Caimari, et al. 2010b; Radler, et al. 2011), fasting (Caimari, et al. 2010a; Oliver, et al. 2013) and to weight reduction strategies (de Mello, et al. 2008).

All in all, considering that PBMC gene expression profile may reflect the gene expression profile of liver, adipose tissue and muscle, and taking advantage of their easy accessibility and their exposure to such a variety of organ systems, PBMCs provide an attractive target tissue and an interesting source of biomarkers of health in the field of human nutrigenomics.

4.2. Quantitative screening of protein damage biomarkers

The term of protein damage biomarkers includes glycation, oxidation and nitration adducts. The accumulation of these adducts in biological systems represents a new type of metabolic imbalance that is providing improved understanding of disease and health decline in ageing.

Among adducts of protein damage, advanced glycation endproducts (AGEs) are of special interest. AGEs are yellow-brown fluorescent and insoluble adducts that are formed endogenously in body tissues by protein glycation reactions. Interestingly, their formation progressively increases with normal ageing; however, their formation can be even significantly increased by several pathological conditions, such as diabetes. Diabetic patients, as consequence of the high glucose and other saccharide derivatives levels in plasma, display a raised AGE formation and hence an increased level of glycation of various structural and functional proteins including plasma proteins and collagen (Ahmed and Thornalley 2007; Singh, et al. 2014). AGEs are accumulated intracellularly on long-lived proteins playing important roles as stimuli for activating intracellular signalling pathways, as well as modifying the function of intracellular proteins and even modifying the functions of the whole cell. On the one hand, AGEs impair the physiological functions of proteins, which undergo accelerated degradation to free AGEs adducts. AGEs-modified proteins lose their specific functions by disrupting molecular conformation, altering enzymatic activity, reproducing degradation capacity, and interfering with receptor recognition. On the other hand, glycation of proteins can also alter the cell functions. The mechanisms by which AGEs modify the cell functions include denaturation and functional decline of the target protein or lipid, organopathy due to accumulation of AGEs in the tissue, activation of receptor mediated signal pathway in cells, and generation of oxidative stress and carbonyl stress [reviewed by (Singh, et al. 2014)].

Physiologically, there is a ubiquitous enzymatic defence against glycation which consists of enzymes that repair early glycated proteins and prevent glycation by metabolizing dicarbonyl

glycating agents. The imbalance between glycating agents and enzymatic defence against glycation in favour of glycating agents has been termed carbonyl stress and is thought to contribute to ageing and disease (Xue, et al. 2009). In the clinical setting, increased formation and steady-state accumulation of glycated proteins have been linked to disease development and progression, particularly diabetes and its vascular complications. Furthermore, formation and accumulation of AGEs have also been linked with renal failure (Agalou, et al. 2005) and with cardiovascular and Alzheimer's diseases (Ahmed, et al. 2005; Thornalley, et al. 2003). In addition, there is substantial evidence linking glycation damage protein to obesity and pro-oxidative and pro-inflammatory events (Klenovics, et al. 2013; Thornalley, et al. 2003). Involvement of AGEs in arthritis and cirrhosis (Ahmed, et al. 2004), anxiety and Schizophrenia (Arai, et al. 2010), and other diseases is emerging.

Concerning AGEs formation (**Figure 4**), protein glycation reactions occurs by complex series of sequential and parallel non-enzymatic reactions called collectively the Maillard reaction (Rabbani and Thornalley 2012). This reaction is subdivided into three stages. In the early stage, carbonyl groups of glucose or other reducing sugars react with free amino groups of proteins, leading to the formation of glycosylamine which dehydrates to form an unstable compound, the Schiff base. The Schiff's base undergoes an Amadori rearrangement to form N-(1-deoxy-D-fructos-1-yl)amino acids or fructosamine. Glycosylamine, Schiff's base and fructosamines are considered to be early stage glycation adducts [reviewed by (Rabbani and Thornalley 2012)]. In an intermediary stage, the amadori products degrade to a variety of reactive dicarbonyl compounds or α -oxoaldehydes such as glyoxal, methyl-glyoxal and deoxyglucosones via dehydration, oxidation and other chemical reactions. In the late stage, the reactive dicarbonyls react irreversibly with amino groups of amino compounds to form AGEs through oxidation, dehydration and cyclization reactions [reviewed by (Singh, et al. 2014)]. Moreover, the reactive dicarbonyls formed during the intermediary stage of Maillard reaction can also react with proteins to directly form AGEs. The Schiff's base adducts can also degrade via non-Amadori rearrangement reaction pathways to α -oxoaldehydes which also leads to the formation of AGEs. The latter are also formed by the direct modification of proteins by α -oxoaldehydes produced by the degradation of glycolytic intermediates and lipid peroxidation.

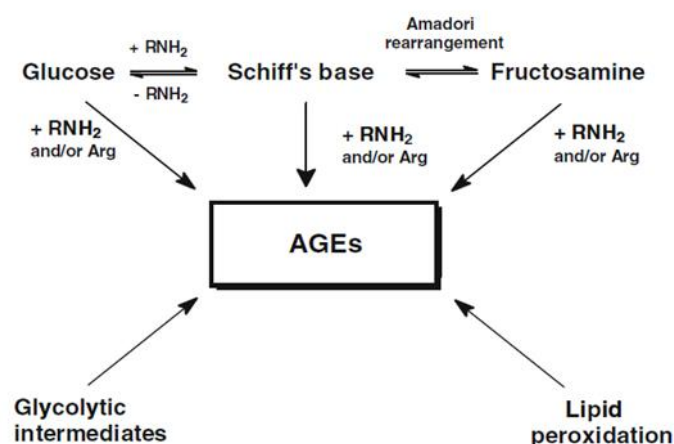


Figure 4. Early glycation and sources of advanced glycation endproducts (AGEs) (Rabbani and Thornalley 2012)

Long-lived proteins contain numerous lysine, hydroxylysine and arginine residues that are prone to age-related accumulation of glycation damage. In this regard, some free glycation adducts derived from arginine residues are: N ω -carboxymethylarginine (CMA), N δ -(5-hydro-4-imidazol-2-yl)ornithine (G-H1), Glucosepane, N δ -(5-hydro-5-methyl-4-imidazol-2-

yl)ornithine (MG-H1), 3-deoxyglucosone (3DG-H) and Ornithine (Ahmed and Thornalley 2007). The glycation adducts derived from lysine residues are: N ϵ -carboxyethyl-lysine (CEL), N ϵ -carboxymethyl-lysine (CML), Fructosyl-lysine (FL), bis(lysyl) crosslink derived from methylglyoxal (MOLD) and Pyrraline. Further classification of AGEs has been made to relate to the mechanisms of AGE formation (**Figure 5**). Major glycation adducts in physiological systems are formed by non-oxidative processes. They include hydroimidazolones (G-H1, MG-H1 and 3DG-H), Monolysine adducts (CEL and Pyrraline), Imidazolium and non-fluorescent crosslinks (MOLD and Glucosepane) and other structures such as CMA. Some AGEs are formed by oxidative processes and are called “glycooxidation products”. Examples of glycooxidation products are pentosidine and CML (Rabbani and Thornalley 2012).

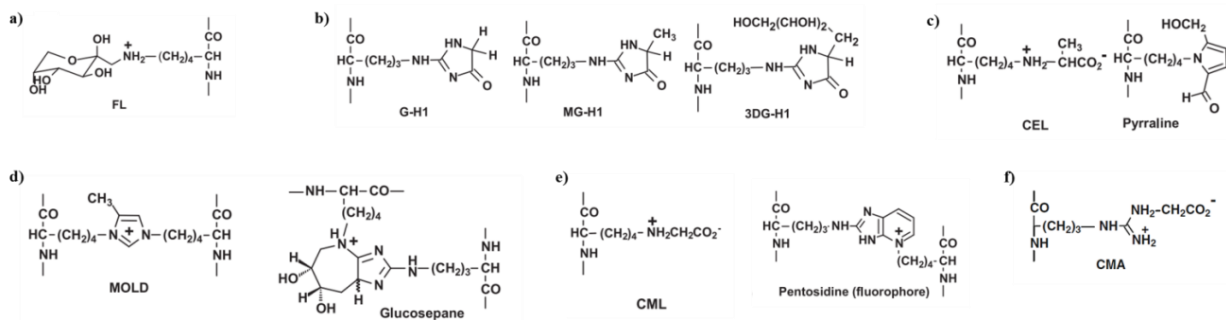


Figure 5. Molecular structures of protein glycation adduct residues. **a**) Early glycation adducts formed by glycation with glucose. **b-f**) Advanced glycation endproducts (AGEs). **b**) Hydroimidazolones. **c**) Monolysine adducts. **d**) Imidazolium and other non-fluorescent crosslinks. **e**) Glycooxidation products, and **f**) Other structures. For the corresponding free adducts at physiological pH, the N-terminal amino group is protonated $-\text{NH}_3^+$ and the C-terminal carbonyl is a carboxylate $-\text{CO}_2^-$ moiety. Adapted from (Thornalley and Rabbani 2014).

Despite AGEs are formed endogenously in physiological systems, protein glycation reactions also occurs in culinary processing, particularly during heat treatment of saccharide-rich food and alkaline treated bakery products (Singh, et al. 2014). The content of glycation protein adducts in food, their bioavailability, clearance and residence time in the body, as well as their functional effects, still remain controversial. Therefore, the contribution of glycation adducts from food to total physiological exposure to glycation adducts remains a critical issue for further investigation [(reviewed by (Rabbani and Thornalley 2012)].

It has been demonstrated in rats that AGEs from food are absorbed by the intestines into circulation and are eliminated from the organism, as reflected by the increase in their kidney content and urinary excretion (Somoza, et al. 2006). In fact, in healthy humans, urinary excretion of AGEs is considered to be strictly dependent on daily diet (Forster, et al. 2005). Several lines of evidence favour the concept that exaggerated intake of thermally processed food might induce pathogenic pathways or aggravate a pre-existing pathology. In this sense, human studies have revealed that there are significant correlations between ingested AGEs, circulating AGEs, and several markers of inflammation (Vlassara 2005). Strikingly, at least in experimental animals, high consumption of heat treated foods may promote the manifestation of diabetes, and substantially contribute to the acceleration of the development of diabetic complications (Sebekova and Somoza 2007). In addition, animal studies suggest that feeding an AGE-rich diet may accelerate chronic kidney disease progression and renal fibrosis via a redox-sensitive inflammatory pathway (Sebekova and Somoza 2007). Therefore, highly thermally modified food may exert, in rodents and humans, various metabolic and nephrotoxic

effects, aggravating inflammation and oxidative stress. Thus, the recommendation of avoiding high intakes of severely thermally treated foods may be of clinical relevance and can possibly result in an extended healthy life span (Vlassara 2005).

Concerning protein oxidation and nitration, are also implicated in some diseases (Finkel and Holbrook 2000; Greenacre and Ischiropoulos 2001). Concretely, oxidative protein adducts are formed by the reaction of peroxides, hypochlorite, oxidizing free radicals and heme iron species with susceptible amino acids, particularly methionine and tyrosine, and cysteine thiols, tryptophan and histidine. These reactions may be increased by oxidative stress arising from mitochondrial dysfunction, activation of NADPH oxidase, uncoupling of nitric oxide synthase, metabolism of environmental chemicals and drugs, and many other processes (Chance, et al. 1979; Horiuchi 1996). Concerning protein nitration, it is increased in cellular-mediated immunity, vascular disorders, endotoxaemia and other diseases, particularly when there is high expression of inducible nitric oxide synthase (Greenacre and Ischiropoulos 2001). The major protein nitrosation biomarker is 3-nitrotyrosine (3-NT), formed by the reaction of tyrosine with peroxynitrite and chlorylnitrite (Greenacre and Ischiropoulos 2001). Other important protein oxidation biomarkers are: Glutamyl semialdehyde (GSA), Methionine sulfoxide (MetSO) and N-formylkynurenine (NFK) (Rabbani and Thornalley 2009) (**Figure 6**).

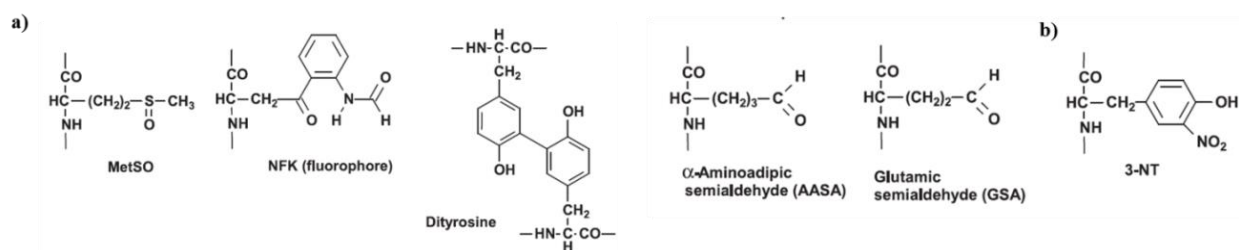


Figure 6. Molecular structures of protein oxidation (a) and nitration (b) adduct residues. For the corresponding free adducts at physiological pH, the N-terminal amino group is protonated $-\text{NH}_3^+$ and the C-terminal carbonyl is a carboxylate $-\text{CO}_2^-$ moiety. Figure adapted from (Thornalley and Rabbani 2014).

In summary, biological proteins can be damaged, endogenously, by glycation, oxidation and nitration reactions throughout life (Karachalias, et al. 2010). Moreover, these reactions can be even significantly increased by several pathological conditions, such as diabetes and other metabolic diseases. Due to the fact that glycation, oxidation and nitration reactions are implicated in protein misfolding, the modified proteins lost their normal functions and are finally degraded by the proteasome (Thornalley 2008). As a result, free amino acids and free glycation, oxidation or nitration adducts, are released into the bloodstream (Thornalley 2005) and are considered to be potential biomarkers of protein damage (Thornalley, et al. 2003). Therefore, identification of free amino acids, and free glycation, oxidation and nitration adducts in biological samples from individual exposed to specific nutritional environments during critical periods of their development could throw light upon which factors, due to this nutritional condition, could affect the future metabolic health of an individual.

Chapter II.

Sim and experimental design

Aim and Experimental design

Obesity has become a worldwide health problem, particularly in developed countries where its prevalence has increased substantially over the last decades. Moreover, obesity also increases the risk of insulin resistance, type 2 diabetes, hypertension and cardiovascular disease. As consequence, obesity has received considerable attention from the media and from the scientific community, who is trying to develop and implement therapeutic or preventive strategies to combat the global burden of obesity and its related metabolic alterations.

The rising rate of obesity has been long attributed to a direct consequence of modern life's access to large amounts of palatable, high calorie food and limited physical activity, together with the genetic background. However, many studies in human and animal models suggests that obesity prevalence and its raise in last decades may also be associated to complex environmental, behavioural and genetic influences during prenatal and postnatal stages of life, which ultimately may lead to a chronic energy imbalance that favours energy accumulation and excessive weight gain (Guilloteau, et al. 2009; Martin-Gronert and Ozanne 2013; Martorell, et al. 2001). In this regard, maternal nutritional environment, such as food restriction, during perinatal stages may program the risk in their offspring to develop obesity and related metabolic pathologies in adult life. Therefore, considering that obesity and its related metabolic alterations can be programmed already at early stages of life, understanding the molecular mechanisms underlying this developmental programming becomes crucial. Identification of these mechanisms could make possible the development of new strategies for reverting or preventing the programmed obesity and related metabolic alterations; they may also help in the identification of early biomarkers which could predict the propensity to develop obesity at very early stages of life, before the phenotypic features become evident. Identification of such biomarkers, particularly those related with improved metabolic health, becomes very interesting because they may also be used as tools to determine the effectiveness of nutritional intervention strategies during critical periods of development directed to program a greater protection against obesity and its related metabolic disorders in adult life.

Therefore, the main aim of the present PhD Thesis was:

To characterize in rats the programming effects of moderate maternal energy restriction during pregnancy or lactation on the metabolic health of their offspring in terms of obesity and related metabolic alterations, as well as to identify new preventing strategies against programmed obesity and early biomarkers of metabolic health.

The experimental work 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). The PhD project has been possible thanks to a pre-doctoral research fellowship from the Spanish Government, (FPU, “Formación de Profesorado Universitario”). The research has been funded mainly by the European Union project “BIOCLAIMS” (FP7-244995) and also by the national projects: “BIOBESMARKERS” (AGL2009-11277) and “EPIMILK” (AGL2012-33692).

In addition, to advance in the development of this research project and to broaden the PhD candidate formation, I performed two research periods of 3 months each one (from the 21st of September, 2012 until the 21st of December, 2012; and from the 19th of August, 2013 until 18th of November, 2013) in the laboratory of Professor Paul Thornalley of the Division of Metabolic and

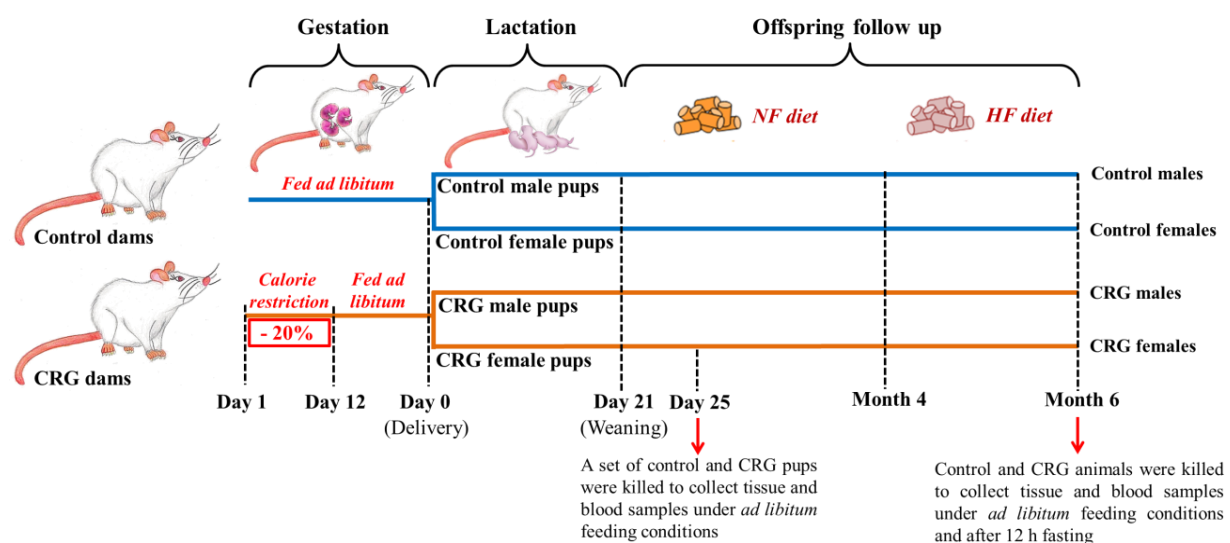
Vascular Health, Warwick Medical School at University of Warwick (Coventry, United Kingdom). These training periods abroad were performed within the framework of the BIOCLAIMS and EPIMILK projects, and were partially funded by the CIBEROBn. The purpose of these stages was to learn the technique of liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) and to apply it for the analysis of free amino acids and protein damage markers in milk and plasma samples.

In order to achieve the principal aim of this PhD Thesis four specific objectives have been established, and for each of them several specific tasks have been undertaken.

1. To determine the mechanisms underlying the higher susceptibility to obesity and related metabolic alterations of the offspring of rat dams exposed to food intake restriction during pregnancy, and to test whether an experimentally induced increase of hepatic fatty-acid oxidation (FAO) in these animals could be considered as a strategy to reverse the metabolic disturbances related to developmental programming.

Previous studies in our laboratory showed that maternal calorie restriction of 20% during the first 12 days of gestation has negative outcomes in the adult offspring. In particular, males and females showed higher food intake than their controls and this resulted in higher body weight in adulthood, but only in male animals. These results suggested that maternal calorie restriction during gestation has different lasting outcomes on offspring's body weight depending on the gender (Palou, et al. 2010a). Hence, the specific objective was to identify potential mechanisms involved in these programming effects and to test one concrete strategy to reverse, at least in part, these adverse effects. Two tasks were set out in order to achieve the first specific objective:

Task 1.1. To study the effects of moderate maternal calorie restriction during pregnancy on determinants of later leptin and insulin resistance, and the different programming effects between male and female animals. To perform this, two groups of male and female rats were studied: the offspring of control dams and the offspring of dams subjected to moderate calorie restriction (20%) during the first 12 days of pregnancy (CRG animals).

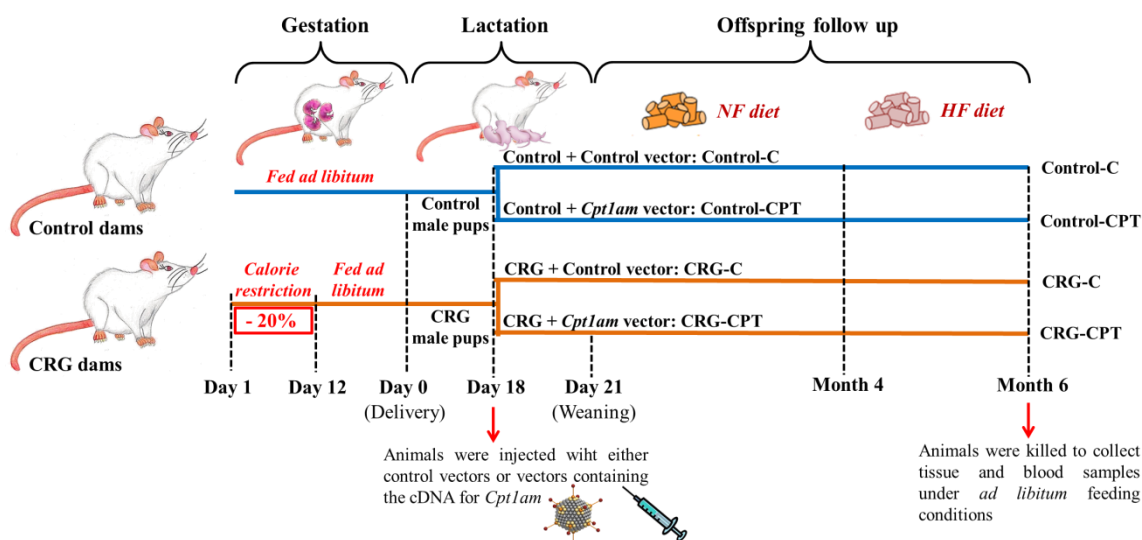


Body weight and food intake of control and CRG animals were measured from delivery until the age of 6 months. All animals were fed with standard diet until the age of 4 months, and then, all of them were exposed to a HF-diet (4.7 kcal/g, with 45% calories from fat) until the age of 6 months.

The expression of energy balance-related genes (including insulin, leptin and their respective receptors) in hypothalamus, retroperitoneal white adipose tissue (rWAT) and liver were analysed both at juvenile age (25 days old; to determine whether this dietary stressor during gestation was able to step up early programmed disorders) and in adulthood (6 months old). Moreover, considering the important role of leptin during perinatal development, we also investigated whether the effects of maternal calorie restriction during gestation on later leptin and insulin homeostasis could be related with alterations in leptin levels during the suckling period. To achieve this, blood samples from pups were collected at different stages of lactation (days 6, 9 and 15 of lactation) under *ad libitum* feeding conditions to obtain plasma for leptin determination. Furthermore, insulin and leptin levels were measured in plasma samples from 25-day- and 6-month-old animals. HOMA-IR was also determined at the age of 6 months. Results from this study are included in the **manuscript 1**.

Task 1.2. To study whether increased hepatic FAO through AAV-mediated gene transference of *Cpt1am* at juvenile ages may prevent or revers the metabolic negative outcomes observed in the offspring of 20% maternal calorie restriction during gestation due to developmental programming.

There is evidence in the literature showing that, in obese mice, a permanent increase of hepatic FAO by injection of AAV vectors containing the key gene for mitochondrial β -oxidation, the *Cpt1a* or *Cpt1am* (which encodes for a permanently active form of CPT1A insensitive to its physiological inhibitor malonyl-CoA), leads to a reduction of obesity-induced hepatic steatosis, weight gain, inflammation, diabetes and insulin resistance in obese mice consuming HF-diet. Therefore, considering this and to accomplish the task 1.2, we used a new cohort of control and CRG male animals. AAV vectors were administered by tail vein injection in 18-day-old control and CRG male pups. Half of the male control and CRG pups were injected with control AAV vectors (Control-C and CRG-C animals, respectively); the other half of pups were injected with AAV vectors carrying the cDNA for *Cpt1am* (Control-CPT and CRG-CPT animals, respectively).

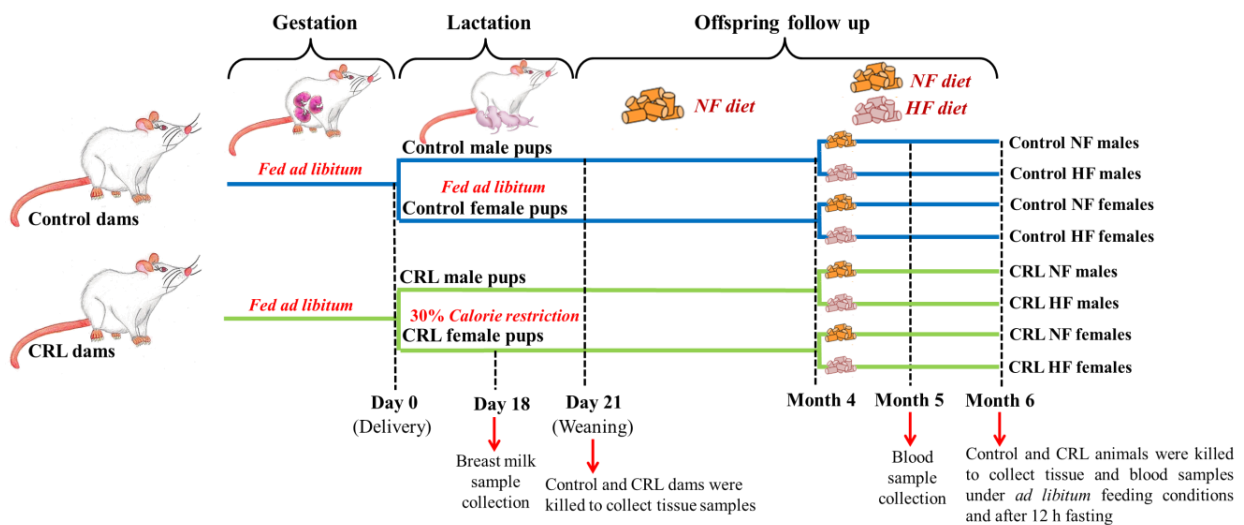


Body weight and food intake of control and CRG animals were recorded from delivery until the age of 6 months. All animals were fed with standard diet until the age of 4 months, and then, all of them were exposed to a HF-diet until the age of 6 months. Locomotive activity and energy expenditure were measured at the age of 3 months by indirect calorimetry. Blood pressure was

also measured at the age of 5 months. Finally, at the age of 6 months, animals were killed to collect liver, rWAT and blood samples. Gene expression analysis of liver, as well as morphological, histological and biochemical analysis of liver and rWAT were performed. Results from this study are included in the **manuscript 2**.

2. To characterize the effects of food intake restriction of rat dams during lactation on the offspring predisposition to obesity and related metabolic disorders, as well as to identify potential mechanisms involved in the early postnatal nutrition programming.

Maternal calorie restriction during gestation is already known to have long lasting negative effects on their offspring, which become programmed for a higher propensity the develop obesity and related metabolic alterations in a gender depended manner. However, the lasting effects of early postnatal nutrition, particularly moderate maternal calorie restriction during lactation, are still uncertain. Several animal studies have been previously performed focusing on severe maternal energy restriction and/or protein restriction during lactation, or by increasing the litter size during the suckling period. In general, these conditions showed protective effects against overweight in the offspring, however, some detrimental effects could also be observed on the normal development of these animals. Therefore, in order to fulfil the second specific objective, two groups of male and female animals were studied: the offspring of control dams and the offspring of dams exposed to moderate calorie restriction (30%) throughout the whole lactating period (CRL animals).



All animals were fed with standard diet until the age of 4 months; then, half of them continued with standard diet (normal fat – NF – animals), and the other half was fed with HF-diet (HF animals) until the age of 6 months.

Hence, two tasks were performed:

Task 2.1. To study the effects of 30% moderate maternal calorie restriction during lactation on later body weight, insulin sensitivity and other energy homeostasis-related parameters of their adult male and female offspring. To achieve this, body weight and food intake of control and CRL animals were recorded from delivery until the age of 6 months. Circulating parameters were also measured in plasma of 5-month-old offspring. Additionally, we determined whether leptin content in maternal milk or leptin production by the mammary gland could be affected by maternal calorie restriction during lactation. To accomplish this, milk samples were collected from control and

CRL dams at day 18 of lactation, and milk leptin levels were measured. mRNA expression levels of leptin were also determined in mammary gland of dams. Results from this study are included in the **manuscript 3**.

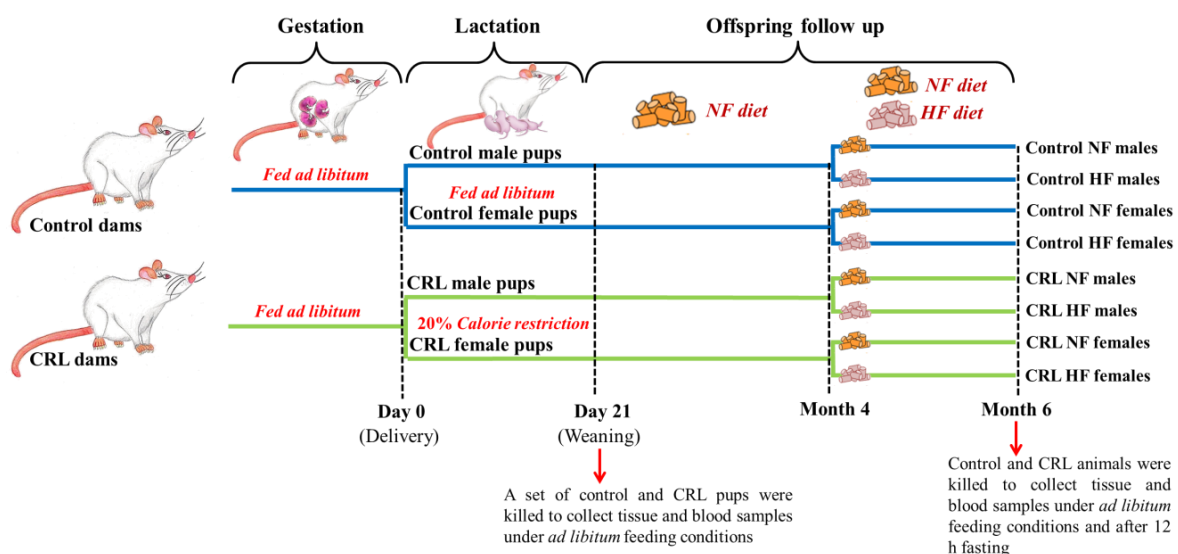
Task 2.2. To gain further insight into the mechanisms that could underlie the beneficial outcomes observed in the adult offspring of dams subjected to 30% moderate calorie restriction during lactation. To achieve this, mRNA expression levels of selected genes involved in the regulation of food intake (in the hypothalamus) and fat accumulation (in rWAT) were analysed. In addition, circulating parameters of 6-month-old control and CRL animals, under NF and HF-diet, were analysed under feeding conditions (and also under fasting conditions for HF-diet exposed animals). The relationship between mRNA expression levels of those genes analysed and energy homeostasis-related parameters under HF-diet feeding conditions in adulthood was also examined. Results from this study are included in the **manuscript 4**.

3. To use the offspring of dams exposed to food intake restriction during lactation, as an animal model programmed for a lower propensity to develop obesity and related metabolic alterations, for identifying early transcriptome-based biomarkers of metabolic health.

In previous tasks (2.1 and 2.2), we studied the effects of 30% maternal calorie restriction during lactation on the offspring metabolic health in adult life. Here we analysed whether a less severe maternal calorie restriction (20%) during lactation, which could be a typical situation of dieting representative or applicable in humans, also causes beneficial effects in offspring against obesity development and other metabolic alterations in adult life. If so, we planned to use this model as a tool to identify early biomarkers of metabolic health.

In order to accomplish this specific objective, two groups of male and female animals were studied: the offspring of control dams and the offspring of dams subjected to 20% moderate calorie restriction throughout the whole lactating period (CRL animals).

All animals were fed with standard diet until the age of 4 months; then, half of them continued with standard diet (NF animals), and the other half was fed with HF-diet (HF animals) until the age of 6 months.



Two tasks were developed:

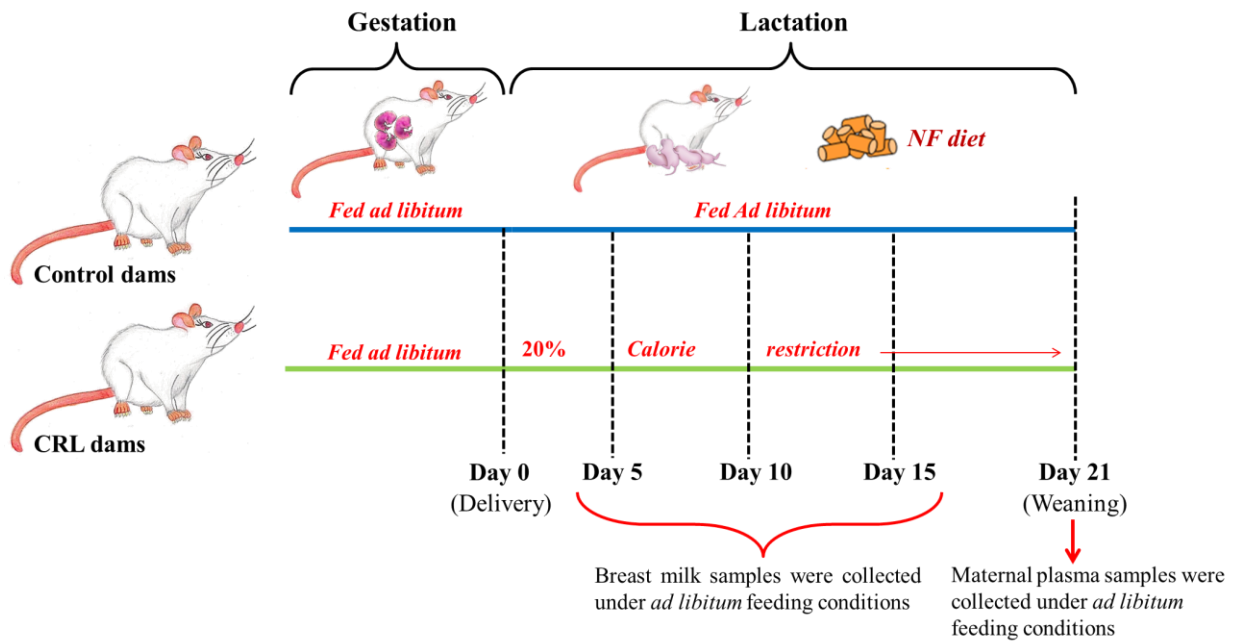
Task 3.1. To characterise the effects of 20% maternal calorie restriction during lactation in the offspring, in terms of body weight and metabolic health. To achieve this, body weight and food intake of control and CRL animals were recorded from delivery until the age of 6 months. Circulating parameters were also measured at weaning (postnatal day 21) and at the age of 6 months. In addition, we investigated early adaptations at the transcriptional level in key tissues involved in energy homeostasis, such as rWAT and liver, and whether these changes are maintained in adulthood (6 months old) under HF-diet exposure. Results from this study are included in the **manuscript 5**.

Task 3.2. To use the animal model consisting in the offspring of 20% calorie restricted dams during lactation to identify early predictive biomarkers of metabolic health by transcriptome profiling of PBMCs samples. We also studied whether the potential biomarkers identified at juvenile age continue to serve as potential biomarkers in adulthood, and whether the changes occurring in blood cells reflect the metabolic environment in key tissues. To achieve this task, whole-genome microarray analysis of PBMCs samples from 21-day-old control and CRL pups were performed. To validate microarray analysis, expression patterns of selected genes related to lipid metabolism were fully confirmed by Real-Time quantitative Polymerase Chain Reaction (RT-qPCR). Transcript levels of these genes were also assessed by RT-qPCR in PBMCs from 6-month-old adult rats and in relevant metabolic tissue samples, such as liver and WAT, of 21-day- and 6-month-old animals. Results from this study are included in the **manuscript 6**.

4. To determine whether the beneficial effects observed in the offspring of moderate calorie restricted lactating rat dams might be related with changes in free amino acid levels and protein damage markers in maternal plasma and breast milk.

Maternal nutritional environment during the lactating period may result in changes in milk composition. Such changes could be modifications in the integrity of specific components contained in breast milk, throughout reactions of glycation, oxidation or nitration reactions. These reactions generate, in turn, misfolded proteins that are degraded into free amino acids and free protein damage adducts, including glycation, oxidation or nitration free adducts. Thus, the analysis of free protein damage adducts and of free amino acids, in breast milk and plasma samples of mothers exposed to moderate food restriction during lactation, could throw light upon the mechanisms that can affect the future metabolic health of an individual, as consequence of changes in its nutritional environment during lactation. To accomplish this, the following task was performed:

Task 4.1. Quantitative screening of free amino acids and of a comprehensive range of free glycation, oxidation and nitration adducts in breast milk and plasma samples from control and 20% calorie restricted dams during lactation. To achieve this, two groups of lactating rat dams were studied: control dams and dams subjected to moderate calorie restriction (20%) throughout the lactating period (CRL dams).



Breast milk samples —collected at days 5, 10 and 15 of lactation— as well as, maternal plasma —collected at day 21 of lactation— from control and CRL dams were analysed by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS). Results from this study are included in the **manuscript 7**.

Chapter III.

*M*aterials and methods

Materials and methods

1. Experimental animals

All the studies were conducted on male and female *Wistar* rats purchased from Charles River Laboratories (Barcelona, Spain). During experiments, animals were housed under standard conditions in the animal house at University of the Balearic Islands (UIB): controlled temperature (22°C), 12 h light-dark cycle and, free access to tap water and standard laboratory rodent chow diet (3 Kcal/g, with 8% calories from fat; Panlab, Barcelona, Spain), unless specify.

Body weight and food intake of animals were recorded. Body length was measured from the tip of the nose to the anus. Body composition was determined by using an EchoMRI-700TM (Echo Medical Systems). All these measures were performed without anesthesia.

In some studies it was necessary to collect tissue samples from juvenile animals. Hence, in these experiments a set of animals were killed at juvenile ages, either at weaning (21 days old) or at the age of 25 days, while the other set of animals was killed in adulthood (at the age of 6 months). Animals were killed by decapitation during the first 2 h at the beginning of the light cycle, under *ad libitum* feeding conditions or after 12 h fasting, depending on the experimental design. Selected tissues, such as hypothalamus, liver, mammary gland and white adipose tissue depots (gonadal, inguinal, mesenteric and retroperitoneal; gWAT, iWAT, mWAT and rWAT, respectively) were rapidly removed, weighed, washed with 0.1% diethyl pyrocarbonate (DEPC) saline solution (to avoid biochemical and molecular tissue degradation), frozen in liquid nitrogen and stored at -80°C until posterior analysis.

Blood samples were collected from all animals at different time-points (at 2, 4 and 6 months of life) and under *ad libitum* feeding conditions and/or under 12 h fasting conditions. At 2 and 4 months, blood samples were collected in heparinized containers from the saphenous vein, without anesthesia and during the first 2 h at the beginning of the light cycle. When animals were killed, trunk blood samples were collected in heparinized containers, after animal decapitation, for plasma and peripheral blood mononuclear cells (PBMCs) isolation. Plasma was obtained by centrifugation of heparinized blood at 1000 x g for 10 min at 4°C. Plasma was saved in new sterile containers and stored at -20°C until posterior analysis.

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

Reagents:

- Standard chow diet (Panlab).
- 0.1% DEPC-saline solution: 1 mL of DEPC (Sigma) in 1 L of saline solution.

2. PBMCs isolation

PBMCs, a subset of white blood cells including lymphocytes and monocytes, were isolated from the samples of trunk blood by density gradient separation using the medium Ficoll-Paque™ PLUS (GE Healthcare).

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

The medium Ficoll-Paque™ PLUS (GE Healthcare) is a shallow density gradient of Ficoll sodium diatrizoate solution with a proper density of 1.077 g/mL. In addition to its viscosity, and osmotic pressure, this medium allows lymphocyte isolation in a simple and rapid procedure, following the instructions of the manufacturer.

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

Reagents:

- 0.2% Heparin in 0.9% NaCl: 0.2 g of heparin (Sigma) in 100 mL of 0.9% NaCl (Panreac)
- Balanced Salt Solution (BSS): mixture 1:9 of solution A and solution B. Solution A: 5.5 mM anhydrous D-glucose (Merck), 5 mM CaCl₂ x 2H₂O (Pancreac), 0.98 mM MgCl₂ x 6H₂O (Pancreac), 5.4 mM KCl (Pancreac), 145 mM Trizma base (Sigma), pH adjusted to 7.6. Solution B: 140 mM NaCl (Pancreac).
- Ficoll-Paque™ PLUS (GE Healthcare)

3. RNA isolation and quantification

Total RNA was isolated from hypothalamus, liver, mammary gland, iWAT and rWAT by commercial Tripure Reagent Method (Roche Applied Science) or by E.Z.N.A. Total RNA kit I (Omega Bio-Tek), depending on the type of tissue and its size. In general, when the entire tissue needed to be homogenized for gene expression analysis, such as hypothalamus, or the size of tissue was too small (especially tissue samples collected from young animals), the extraction was performed following the Tripure Reagent Method, according to the manufacturer's instructions. For tissue samples collected from adult animals (relatively larger size of tissue) or when the intended use of extracted yield required its high quality and purity (for example to use RNA from PBMCs for microarray analysis), total RNA was extracted using E.Z.N.A. Total RNA kit I, according to the manufacturer's instructions.

3.1. Tripure reagent method for RNA isolation

The whole hypothalamus or approximately 100 mg of WAT, liver or mammary gland were homogenized in ice, in 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 the RNA integrity and denatures endogenous nucleases, while disrupting cells and dissolving cell components.

In case of WAT, liver and mammary gland, to remove the fat fraction, homogenates were centrifuged at 12000 x g for 10 min at 4°C, 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 x g for 15 min at 4°C was performed to generate three phases: a colorless aqueous upper phase which contains RNA, a white interphase with DNA and an organic pink phenol-chloroform lower phase which contains proteins (which was stored at -20°C). 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°C. Afterwards, samples were centrifuged at 12000 x g for 10 min at 4°C to precipitate RNA. Isopropanol was removed and the precipitated RNA was washed with 1 mL of 75% ethanol, vigorously shaken and centrifuged at 7600 x g for 5 min at 4°C to remove ethanol completely. To increase the efficiency and have a major purity of RNA, the ethanol wash step was performed twice. Finally, ethanol was completely removed and 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°C.

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 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 or, 10 mg of WAT or liver, were homogenized in 350 µl of TRK Lysis Buffer (700 µl for WAT) 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. β-mercaptoethanol reduces RNA degradation. To remove the fat fraction, homogenates were centrifuged at 13000 x g for 5 min at 4°C. Cleared supernatant was transferred carefully into a fresh sterile tube and 350 µl (or 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 x g for 2 minutes at room temperature. The flow-through was discarded. Then, samples were washed with 350 µl of RNA Wash Buffer I, centrifuged at 10000 x 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 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 x 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 x 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 x g for 1 min at room temperature and discarded the flow-through. Then, to completely dry the HiBind membrane, columns were centrifuged, with the collection tube

empty, at maximum speed (20000 x 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 DEPC-treated water (added directly onto the center of the membrane's column) by centrifuging at 12000 x g for 4 min at room temperature.

3.3. RNA quantification and assessment of its integrity

Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). This spectrophotometer is used for quantification of nucleic acids by using UV absorption. The concentration of the nucleic acid can be determined using the Beer-Lambert law, which predicts a linear change in absorbance with concentration. RNA has its maximum absorption at 260 nm, therefore, absorption at 260 nm of 1.0 is equivalent to about 40 µg/ml of RNA and hence, the optical density at 260 nm is used to determine the RNA concentration in a solution. NanoDrop ND-1000 spectrophotometer also determines the ratio of absorbance at 260 nm and 280 nm to assess the purity of RNA. A ratio of ~ 1.8 is generally accepted as “pure” for DNA; a ratio of ~ 2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of proteins that absorb strongly at or near 280 nm. If the ratio for RNA is appreciably upper than 2, it determines contamination with DNA. Indeed, this spectrophotometer also determines a secondary ratio of absorbance at 260 nm and 230 nm, which indicates the contamination degree with organic solvent. Expected 260/230 values for “pure” nucleic acid are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants, which absorb at 230 nm, such as organic solvents. For spectrophotometric quantification of isolated total RNA, 2 µl of sample were used.

Verification of integrity of isolated RNA was confirmed using 1% agarose gel electrophoresis. Electrophoresis technique relies on the negative charge of nucleic acids for size separation in a sieved matrix. Visualization of 28S and 18S ribosomal RNA (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.

Agarose gels were made in 0.5x electrophoresis running buffer and stained with SYBR Safe DNA Gel Staining (1 µl of SYBR Safe DNA Gel Staining for each 10 mL of 0.5x electrophoresis running buffer). RNA samples (250 ng) were mixed with 3 µl of sample loading buffer. Electrophoresis was performed by running the gel at 90 V for 30 min. SYBR Safe DNA Gel Staining has fluorescence maximum excitation at 280 and 502 nm, and a maximum emission at 530 nm. Thus, RNA bands stained with SYBR Safe DNA gel staining were visualized using a UV transilluminator imaging system (ChemiGenius Bio, Syngene). After irradiating the gel with UV light, the emitted fluorescence was photographed using the GeneSnap Software and sharpness of 28S and 18S rRNA bands was evaluated.

Reagents:

- Agarose (Pronadisa)
- β-mercaptoethanol (Sigma)
- Chloroform (Sigma)
- DNAase I digestion reaction mix: 0.75 µl of RNAase-free DNAase I (20 Kunitz/ µl) per each 36.75 µl of E.Z.N.ATM DNase I Digestion Buffer; (Omega Bio-Tek)
- Ethanol absolute (Panreac)
- Glycerol (Sigma)

- Isopropanol (Sigma)
- RNAase-free water (Sigma)
- SYBR Safe DNA Gel Staining (Invitrogene)
- Tripure Reagent (Roche Applied Science)
- E.Z.N.A. Total RNA kit I (Omega Bio-Tek): TRK Lysis Buffer, DEPC-treated water, RNA Wash Buffer I (Omega Bio-Tek) and RNA Wash Buffer II (Omega Bio-Tek).
- 0.5x electrophoresis running buffer: Tris-borate-EDTA buffer (TBE) containing: 44.5 mM Tris base, 44.5 mM Boric acid and 1 mM EDTA
- Sample loading buffer: 50% glycerol (Sigma), 50% water and 2.5 mg/mL bromophenol blue (Panreac)

4. Gene expression analysis by Real-Time quantitative polymerase chain reaction (RT-qPCR)

To measure mRNA expression levels of selected genes, firstly, total isolated RNA was reverse transcribed into complementary DNA (cDNA) and then, the resulting cDNA was amplified and quantified by RT-qPCR.

4.1. Reverse transcription

Isolated total RNA (0.25 µg, in a final volume of 5 µl with RNAase-free water) was denatured for 10 min at 65°C in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem). Then, denatured RNA was reverse transcribed to cDNA after adding 7.5 µl of a reaction mix into in each sample. The reaction mix contained per sample: 1.25 µl of 10x Buffer, 1.25 µl of 25 mM MgCl₂, 2 µl of 2.5 mM dNTPs, 0.5 µl of random hexamers, 0.5 µl of RNAase inhibitor, 0.5 µl of murine leukemia virus reverse transcriptase and 1.5 µl of RNAase free water. The reverse transcription process was performed in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem) following this program: 15 min at 20°C, 30 min at 42°C, 5 min at 95°C and a final step of 4°C indefinitely.

4.2. Real Time quantitative Polymerase Chain Reaction (RT-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 fluorescence from SYBR Green dye meanwhile the PCR amplification process progresses. As the SYBR Green dye binds to double-stranded amplicons, it undergoes a conformational change and emits fluorescence at 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 RNAase 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. RT-qPCR was performed using the Applied Biosystems Step OnePlus™ Real-Time PCR Systems (Applied Biosystems) with the following program: 10 min at 95°C, followed by a total of 40 two-temperature cycles (15 s at 95°C and 1 min at 60°C). To verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle was calculated by the instrument's software (StepOne Software version 2.2). Relative gene expression numbers were calculated as a percentage of control animals, using the 2- $\Delta\Delta C_t$ method (Livak and Schmittgen 2001). Different

housekeeping genes were used as internal controls for gene expression normalization, depending on the tissue, the age and sex of animals, according to their better suitability.

Reagents:

- 50 U/μl murine leukemia virus reverse transcriptase (Applied Biosystem)
- RNAase free water (Sigma)
- 1x Buffer (Promega)
- 2.5 mM MgCl₂ (Promega)
- 100 mM dATP solution (Invitrogen)
- 100 mM dGTP solution (Invitrogen)
- 100 mM dCTP solution (Invitrogen)
- 100 mM dTTP solution (Invitrogen)
- Power SYBER Green PCR Master Mix (Applied Biosystem)
- 50 U/μl random hexamers (Applied Biosystem)
- 20 U/μl RNAase inhibitor (Applied Biosystem)
- Forwards and reverse primers (provided by Sigma)

5. Determination of circulating parameters

5.1. Insulin

Insulin levels were measured in plasma samples using a quantitative two-site sandwich Enzyme-linked Immunosorbent Assay technique (ELISA) applied on the commercial “Rat Insulin ELISA kit” from Mercodia. Insulin levels in plasma samples from young rats (until the age of 1 month) were quantified by using the “Ultrasensitive Rat Insulin ELISA Kit” from Mercodia.

The assay is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. 10 μl of plasma sample or calibrators (25 μl in case of ultrasensitive insulin kit) 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 1+10. During incubation (2 h at room temperature on a plate shaker, 700-900 rpm), insulin in the sample reacted with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies, bounded to micro-titer well. After 5 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)
- Ultrasensitive Rat Insulin ELISA (Mercodia)

5.2. Glucose

Glucose levels were measured in fresh blood using an ACCU-CHEK Aviva system glucometer and test strips from Roche Diagnostics. To achieve this, a fresh blood drop, obtained during blood sample collection from saphenous vein or after killing the animals by decapitation from trunk blood, was applied to the test strip. After waiting some 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 next 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 relative to 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, which is linked to ferrocyanide concentration. Because the ferrocyanide concentration is coupled to glucose concentration, the current measurement permits calculation of blood glucose. Finally, current data are analyzed, and the result is recorded and displayed.

Due to ACCU-CHEK Aviva system uses glucose dehydrogenase chemistry, glucose determination is not affected by varying degrees of sample oxygenation (capillary, venous, alternate site).

5.3. 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 fasting conditions. HOMA-IR was calculated using the formula of Matthews *et al.* previously described (Matthews, et al. 1985)

$$\text{HOMA-IR} = \text{fasting glucose (mmol/L)} \times \text{fasting insulin (mU/L)} / 22.5$$

5.4. Leptin

Leptin levels were measured in plasma and milk samples using a quantitative sandwich enzyme immunoassay technique applied on kit “Quantikine Mouse/Rat Leptin Immunoassay” from R&D Systems.

The microplate provided in this kit is pre-coated with a 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 into their respective wells. Microplate was incubated for 2 h at room

temperature. During incubation process, mouse/rat leptin that is present in the sample bound the immobilized antibody. After removing any unbound substances, by 5 washing steps (by aspiration and washing with Wash Buffer), 100 µl of an enzyme-linked polyclonal antibody specific for mouse Leptin was added into each well. Microplate was incubated for 2 h at room temperature. After 5 washing steps, to remove any unbound antibody-enzyme reagent, 100 µl of substrate solution were added into each well, and microplate was incubated for 30 min at room temperature. During 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 bounded 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).

5.5. Triglycerides (TGs)

Plasma triglyceride levels were determined using an enzymatic colorimetric method applied on “Serum Triglyceride 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 dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H₂O₂)

Subsequently, peroxidase catalyzes the coupling of H₂O₂ with 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (ESPA) to produce a quinoneimine dye that shows an absorbance maximum at 540 nm. The increase in absorbance at 540 nm is directly proportional to 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 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°C and afterwards, the absorbance was read at 540 nm (initial absorbance). After determination of glycerol concentrations, 60 µl of the Triglyceride Reagent were added into each well and the microplate was again incubated for 5 min at 37°C. 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.

Reagents:

- Serum Triglyceride Determination Kit (Sigma)

5.6. Non-esterified fatty acids (NEFAs)

Plasma NEFA levels were measured using an enzymatic colorimetric method applied on kit “NEFA-HR(2) assay” from Wako Chemicals. The procedure involves the enzymatic activity of Acyl-CoA synthetase (ACS), which catalyzes the acylation of coenzyme A (CoA) by the fatty acids. The acyl-CoA thus produced is oxidized by added acyl-CoA oxidase (ACOX) with generation of hydrogen peroxide, that in the presence of peroxidase (POD) permits the oxidative condensation of 3-methyl-N-ethyl-N-(β -hydroxyethyl)-aniline (MEFA) with 4-aminoantipyrine to form a purple colored, 3-Methyl-N-Ethyl-N-(β -Hydroxyethyl)-Aniline (MEHA), which can be measured colorimetrically at 550 nm. The intensity of this pigment is proportional to the concentration of free fatty acids in the sample. Ascorbic acid is removed by ascorbate oxidase from the sample.

This assay was performed according to the manufacturer’s instructions with slight modifications. Firstly, 7 μ l of plasma sample or NEFA standard were loaded into their respective wells. Then, 150 μ l of the Reagent 1, which contains the ACS, were added into each well. After incubating the microplate for 5 min at 37°C, 75 μ l of Reagent 2 were loaded into each well. After incubating the microplate for 5 min at room temperature, the absorbance was measured at 550 nm using a spectrophotometer for microplates (Tecan Sunrise Absorbance Reader).

Reagents:

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

5.7. β -hydroxybutyrate (BHB)

BHB circulating levels were measured using the “ β -hydroxybutyrate determination kit” from Materlab. The principle of this assays focuses on the enzymatic activity of BHB dehydrogenase which catalyzes the oxidation of BHB to acetoacetate with simultaneous reduction of NAD to NADH. The increase of absorbance of NADH is directly proportional to the BHB in the sample. Then, to measure BHB levels in plasma samples, 10 μ l of plasma sample or standard were added into their respective wells and 180 μ l of Working Reagent were also added into each well. After incubating for 10 min at 37°C, samples were excited at 355 nm and the emission of fluorescence was read at 460 nm by using a spectrofluorimeter for microplates (Mithras LB 940). Finally, 30 μ l of Reagent 3 were added into each well and after mixing for 10 min, fluorescence was read again with the spectral properties from above (355 nm excitation and emission at 460 nm).

Reagents:

- β -hydroxybutyrate determination kit (MATERLAB).

5.8. Adiponectin

Plasma adiponectin levels were measured using a quantitative sandwich enzyme immunoassay technique applied on “Rat total Adiponectin/Acrp30 Immunoassay” from R&D Systems. In this assay, a monoclonal antibody specific for rat Adiponectin has been pre-coated onto a microplate. To determine adiponectin levels in plasma, the assay was performed according to the manufacturer’s instructions. Firstly, 50 μ l of Assay Diluent RD1W were added into each

well and 50 μ l 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 incubation process, the immobilized antibody bound any rat adiponectin present in the sample. After 5 washing steps (by aspiration and washing with Wash Buffer), to remove any unbound substances, 100 μ l of Rat adiponectin conjugated (an enzyme-linked polyclonal antibody specific for rat Adiponectin) was added to the wells. After 1 h incubation at room temperature on the shaker, 5 washing steps were performed to remove any unbound antibody-enzyme reagent. Then, 100 μ l of Substrate Solution were added to the wells and 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 μ l 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 a 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 System).

5.9 Tumor Necrosis Factor alpha (TNF α)

Levels of TNF α in plasma samples were measured using a quantitative sandwich enzyme immunoassay technique applied on the “Rat TNF α Immunoassay” from R&D Systems. In this assay, a monoclonal antibody specific for rat TNF α has been previously coated onto a microplate. To quantify TNF α in plasma, the manufacturer’s instructions were followed. Firstly, 50 μ l of Assay Diluent RD1-41 were added into each well and 50 μ l of standard or sample were added into their respective wells. The microplate was incubated for 2 h at room temperature; during incubation process, any rat TNF α present in the sample was bound by the immobilized antibody. After 5 washing steps (by aspiration and washing with Wash Buffer), to remove any unbound substances, 100 μ l of Rat TNF α Conjugated (an enzyme-linked polyclonal antibody specific for rat TNF α) was added to the wells. After 2 h incubation at room temperature, 5 washing steps were performed to remove any unbound antibody-enzyme reagent. Then, 100 μ l of Substrate Solution were added to the wells and microplate was incubated for 30 min at room temperature protected from light. During the incubation process, the enzyme reaction yielded a blue product that turned yellow when 100 μ l of the stop solution were added. The intensity of the color measured is in proportion to the amount of rat TNF α bound in the initial step. Optical density of each well was determined using a spectrophotometer for microplates (Tecan Sunrise Absorbance Reader) set a 450 nm (with wavelength correction at 540 nm). The sample values were then read off the standard curve.

Reagents:

- Quantikine® ELISA Rat TNF α Immunoassay (R&D System).

6. Measurement of hepatic glycogen content

Determination of glycogen was performed following the method of the Anthrone reagent with some modifications. Particularly, for hepatic glycogen isolation, 0.3-0.8 g of liver were

digested and boiled, in test tubes, in 1 mL of 30% KOH at 100°C for 10 min. Then, 2 mL of 100% ethanol were added into each tube and glycogen and proteins were precipitated overnight at -20°C. To remove proteins and collect glycogen, samples were centrifugated at 3000 rpm for 30 min at 4°C. The precipitate was redissolved in 1 mL of 8% trichloroacetic acid (TCA) and then centrifuged at 3000 rpm for 15 min at 4°C. Supernatant was stored at 4°C, whereas the pellet was again redissolved in 1 mL of 8% TCA and centrifuged at 3000 rpm for 15 min at 4°C; the resulting supernatant was added to the stored one. Finally, glycogen was obtained from the supernatant by precipitation with 4 mL of 96% cold ethanol followed by centrifugation at 3000 rpm for 15 min at 4°C. The pellet, containing purified hepatic glycogen, was then redissolved in 1 mL of water and was ready to be quantified by using the Anthrone reagent. 1 mM Glucose solution was used as standard. At this point, 3 mL of Anthrone reagent were delivered into each tube and then boiled at 100°C until the sample color changed from yellow to green-blue (approximately 5 min). Absorbance was read at 590 nm using a colorimeter for test tubes.

Reagents:

- KOH (Panreac)
- Trichloroacetic acid (TCA) (sigma)
- 96% ethanol (Panreac)
- Absolute ethanol (Panreac)
- Anthrone reagent: 0.1% anthrone (Merck) in 84% v/v H₂SO₄ (Panreac)
- Glucose (Merck)

7. Total hepatic lipid content measurement

The extraction of total lipids from liver samples was performed with Folch *et al.* extraction procedure (Folch, et al. 1957).

The liver tissue weighed precisely (0.3 g) was homogenized with 5 mL of chloroform-methanol reagent (2:1 v/v). The exact weight of tissue was recorded for the following calculations. The tissue was disrupted using Potter-Elvehjem homogenizer with a motorized PTFE pestle, and the homogenate was filtrated (funnel with a folded filter paper) to recover the liquid phase. Then, the total volume of the mixture was filled up to 10 mL with chloroform-methanol reagent (2:1 v/v) and the mixture was washed with 2 mL of 0.45% NaCl solution. After dispersion, the whole mixture was agitated vigorously 2 min in an orbital shaker and centrifuged at low speed (3000 rpm) for 10 min at room temperature to separate the two phases. The upper phase (methanol and impurities) was removed, the lower phase (chloroform with lipids) was filled up to 10 mL with pure methanol, and washed with 2 mL of 0.9% NaCl solution. Subsequently, the whole mixture was agitated vigorously 2 min in an orbital shaker and centrifuged at low speed (3000 rpm) for 10 min at room temperature. After centrifugation and siphoning of the upper phase, the lower phase (chloroform with lipids) was filled up to 10 mL with chloroform-methanol reagent (2:1 by volume). 5 mL of the extract was then leaved in a pre-weighted glass vial at 60°C for 24 h to evaporate chloroform. The dry and empty glass vials and vials with lipid extracts were weighed, and the total lipid content was calculated using the following formula:

$$\text{Lipid content [mg/g liver]} = [(\text{extract weight [mg]} / \text{extract weight [mL]}) \times 10] / \text{sample weight [g]}$$

Where:

- *extract weight [mg]* = (weight of the empty glass vial [g] - weight of the glass vial with the extract after evaporation [g]) x 1000
- *extract weight [mL]* = 5 mL
- *sample weight [g]* = weight of liver sample taken for homogenization (the recommended amount of sample is ~0.3 g, although the exact amount taken was noted).

Knowing the weight of the liver of each animal, the content of lipid in the whole liver was calculated.

Reagents:

- Chloroform/methanol (2/1): 2 parts of chloroform (Sigma) diluted with 1 part of methanol absolute (Panreac)
- 0.45% NaCl: 2.25 g NaCl (Panreac) dissolved in 500 mL of distilled water
- 0.90% NaCl: 4.5 g NaCl (Panreac) dissolved in 500 ml of distilled water

8. Measurement of hepatic triglyceride content

To determine TG levels in liver, an extraction of the lipid fraction was firstly needed. Hepatic lipid extraction was conducted by mixing 50-100 mg of liver with 1 mL of hexane/isopropanol (3:2, v/v). To minimize lipid oxidation, tubes were gassed with nitrogen before being closed. Tubes were then left overnight under orbital agitation at room temperature and protected against direct light. Then, 300 µl of 0.47 M Na₂SO₄ were added into each tube, mixed for 5 min, left for 15 min in orbital agitation and finally, centrifuged at 1000 x g for 10 min at 4°C. The bottom phase (containing isopropanol and other polar components) was discarded, while the upper phase of each tube, which contained lipids dissolved in hexane, was transferred to a clean glass tube. Hexane extract was dried with nitrogen gas.

Dry hepatic lipid extracts were resuspended in 1.2 mL of LPL buffer with 0.1% sodium dodecyl sulfate (SDS). Then, samples were sonicated for 30 s, and tubes were left overnight in an orbital shaker at room temperature, protected from light. On the following day, tubes were cold sonicated with three pulses of 30 s each one, and their TG levels were measured immediately using the commercial enzymatic colorimetric kit “Serum Triglyceride Determination Kit” from Sigma, and following the protocol described in section 5.5.

Reagents:

- Hexane (Panreac)
- Isopropanol (Sigma)
- Nitrogen gas (“Carbueros Metálicos”)
- 0.47 M Na₂SO₄: 33.4 g of Na₂SO₄ (Panreac) in 500 mL distilled water.
- Sodium dodecyl sulfate (SDS) (Panreac)
- LPL buffer: 28.75 mM Pipes (Sigma), 57.4 mM MgCl₂·6H₂O (Panreac) and 0.569 mg/mL bovine serum albumin-fatty acid free (Sigma)

9. Histological analysis

Pieces of fresh tissues, such as liver and retroperitoneal white adipose tissue (rWAT) were collected during dissection after animal killing for histological analysis. To avoid cell damage and to preserve the integrity of their membranes, tissue samples were immediately fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH= 7.4) at 4°C for 24 h. Then, fixed samples were washed and stored in 0.1 M phosphate buffer (pH= 7.4) until posterior analysis.

Before sectioning, fixed samples needed to be embedded in paraffin blocks to provide sufficient external support during sectioning. To perform this, the fixative and water from the tissue were removed by dehydration in 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°C; finally, three-times with absolute ethanol for 60 min). The following day, tissue samples were cleared in xylene (two times 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 melt in the oven, at 60°C overnight, to pick up from the bottle the specific tissue we wanted to cut off. The selected tissue was laid on a cassette base and embedded with paraffin to obtain a paraffin block. Then, 5 µm thick sections of tissues were cut using a microtome and mounted on Super-Frost/Plus slides.

Previously to the staining step, the slides were deparaffinised with xylene and re-hydrated with a series of decreasing concentration of ethanol and finally with distilled water. Then, slides were stained with hematoxylin for 2 min and eosin for 5 min, and finally dehydrated with a series of increasing concentration of ethanol. Stained and dehydrated slides were then cleared with xylene and finally mounted with Eukit.

Liver and rWAT sections were digitalized using a light microscope (Zeiss Acioskop) connected with a digital camera (AxioCam Icc3, Carl Zeiss).

To study the presence of fatty vesicles in liver slides or for measuring the diameter of the adipocytes from rWAT slides, software AxioVision 40V 4.6.3.0 (Carl Zeiss, Imaging Solutions) was used.

Reagents:

- 4% Paraformaldehyde: 40 mL Paraformaldehyde (Sigma) up to 1 L in 0.1 M phosphate buffer pH= 7.4
- 0.1 M phosphate buffer pH= 7.4: 0.2 M phosphate buffer diluted 1:1 in distillate water
- 0.2 M phosphate buffer pH= 7.4: 3.25 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (Panreac), 11.24 g Na_2HPO_4 (Panreac) in 1L
- 50% Ethanol: 92 mL of distillate water for each 100 mL of 96% ethanol (Panreac)
- 75% Ethanol: 28 mL of distillate water for each 100 mL of 96% ethanol (Panreac)
- 96% Ethanol (Panreac)
- Absolute Ethanol (Panreac)
- Xylene (Panreac)
- Paraffin wax (Sigma)
- Hematoxilyn (Panreac)
- Eosin (Panreac)
- Eukit (Panreac).

10. Confocal Microscopy for Green Fluorescent Protein Detection in liver samples

Due to the autofluorescence of paraffin, confocal microscopy requires a different way to prepare slide tissues than light microscopy. Hence, instead to use paraffin, tissue samples were frozen and embedded in O.C.TTM compound, which provides a convenient specimen matrix for cryostat sectioning at temperatures below zero using the cryomicrotome HM 505E (Microm International).

To prepare samples, pieces of frozen liver were firstly fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH= 7.4) at 4°C for 24 h, and washed in 0.1 M phosphate buffer (pH= 7.4) for 24 h. For cryoprotection, samples were immersed in 30% sucrose in PBS buffer 1x for 72 h at 4°C, embedded in O.C.TTM compound on the specimen stage and put into the fast freezing station, for 24 h, in the microtome chamber of the cryostat settled at -25°C (the optimal cutting temperature for liver samples). Fifteen micrometer-thick sections of tissues were cut and mounted on slides. For fluorescent staining, sections were firstly washed in PBS buffer 1x and then, hepatocyte nuclei were stained with blue-fluorescence DAPI dilactate 300 nM in PBS buffer for 5 min and protected from light. Slides were washed several times with PBS buffer 1x and dried. Finally, slides were mounted with Dako Fluorescence Mounting Medium, cover-slipped and protected from light.

Microscopic examinations were conducted with an automated inverted microscope DMI 4000B coupled with Leica TCS SPE Confocal Laser System (Leica Microsystems). The fluorescence spectral properties selected were: for DAPI, 405 nm excitation maximum and emission spectral range from 418 to 468 nm; for GFP, 488 nm excitation maximum and emission spectral range from 500 to 550 nm. Digital images were acquired by scanning with the fluorescence spectral properties described above and the projections of the individual channels were merged in Leica Application Suite Software 2.3.6 to facilitate visualization.

Reagents:

- O.C.TTM compound (Sakura Finetek)
- Blue-fluorescence DAPI dilactate 300 nM (Life Technologies)
- Dako Fluorescence Mounting Medium (Dako Diagnostics)
- 4% paraformaldehyde (Sigma)
- 0.1 M phosphate buffer pH= 7.4: 0.2 M phosphate buffer diluted 1:1 in distillate water
- 0.2 M phosphate buffer pH= 7.4: 3.25 g NaH₂PO₄·2H₂O (Panreac), 11.24 g Na₂HPO₄ (Panreac) in 1L
- 30% sucrose (Panreac)
- PBS buffer 1x pH=7.4: 137 mM NaCl (Panreac), 2.7 mM KCl (Panreac), and 10 mM phosphate buffer

11. Isolation of mitochondria from liver

Hepatic mitochondria were isolated from 2 g of fresh liver that was immediately homogenized in 20 mL of a buffer solution using a glass homogenizer Potter-Elvehjem. Homogenate was then centrifuged at 600 x g for 15 min at 4°C and the supernatant was centrifuged again at 12000 x g for 20 min at 4°C. The pellet was resuspended in 2 mL of buffer solution and centrifuged at 7000 x g for 10 min at 4°C. Finally, the mitochondrial-enriched pellet was resuspended in 1 mL of buffer solution and stored at -80°C until used for Western blot analysis.

Reagents:

- Buffer solution pH= 7.4: 70 mM sucrose (Panreac), 220 mM D-mannitol (Sigma), 2 mM EDTA (Merck), 5 mM HEPES (Sigma)
- Protease inhibitor cocktail (Roche)

12. Western blot analysis

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

Western blot was performed to determine CPT1A, total AKT (serine/threonine protein kinase) and phosphorylated AKT on Serine 473 (pAKT) in homogenates of whole liver. Moreover, Western blot was also performed to determine CPT1A in homogenates of the hepatic mitochondrial fraction.

12.1. Sample preparation*Total protein isolation and quantification from whole liver*

An accurately weighed piece of liver was homogenized at 4°C in 1:10 (w:v) of RIPA Lysis buffer 1x supplemented with protease and phosphatase inhibitor cocktail 1x. Completely homogenized liver was centrifuged at 7500 x g for 2 min at 4°C, and the supernatant, containing proteins, was used for protein analysis. Total hepatic protein content was measured with the Pierce BCA protein assay kit, using 2 Albumin Standard Ampules as a standard. Briefly, 25 µl of sample or standard were loaded into their respective wells of standard ELISA plate, and then 200 µl of Working Reagent were added into all wells. Plate was covered and incubated for 30 min at 37°C. Then, absorbance was read at 562 nm using a spectrophotometer for microplates (Tecan Sunrise Absorbance Reader).

Samples were prepared by solubilizing 60 µg of total protein (for CPT1A quantification) or 50 µg of total protein (for total AKT and pAKT quantification) in Laemmli sample buffer (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.

Total protein isolation and quantification from the hepatic mitochondrial fraction

Mitochondria from liver samples were isolated as described in section 11. Mitochondria were precipitated from the mitochondrial-enriched pellet in buffer solution by centrifuging at 7000 x g for 10 min at 4°C. The pellet was sonicated at 4°C in 150 µl of cellular PBS buffer supplemented with protease and phosphatase inhibitor cocktail 1x. The homogenate was centrifuged at 7500 x g for 2 min at 4°C, and the supernatant was used for protein analysis. Total mitochondrial protein content was measured by the method of Bradford (Bradford 1976), using 0.1% bovine serum albumin (BSA) as a standard (standard curve: 0-10 µg).

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

quantify total amount of proteins in the hepatic mitochondrial fraction, 5 µl of sample or standard were loaded into their respective wells of standard ELISA plate and then 250 µl of Bradford reagent were added into each well. After 2 min incubation at room temperature, the absorbance was read at 595 nm using a spectrophotometer for microplates (Tecan Sunrise Absorbance Reader).

Samples were prepared by solubilizing 40 µg of total protein (for CPT1A quantification in hepatic mitochondrial fraction) in Laemmli sample buffer (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 mM Tris-HCl (Sigma) buffer pH=7.4, 150 mM NaCl (Panreac), 0.25% deoxycholic acid (Panreac), 1% NP40 (Sigma) and 1 mM EDTA (Merck)
- Pierce BCA protein assay kit (Thermo Scientific)
- Laemmli sample buffer: 0.5 M Tris-HCl (Sigma) pH=6.8, 5% SDS (Sigma), 10% Glycerol (Sigma), 5% β-mercaptoethanol (Sigma) and 1% Bromophenol blue (Panreac).
- Cellular PBS buffer pH 7,4: 137 mM NaCl (Panreac), 2.7 mM KCl (Panreac), 10 mM Na₂HPO₄ (Panreac), 1.5 mM KH₂PO₄ (Panreac)
- Bradford reagent: 100 g Coomassie brilliant blue G-250 (Merck), 100 mL 85% orthophosphoric acid (Panreac), 50 mL absolute ethanol (Panreac) up to 1L of distillate water.
- 0.1% Bovine Serum Albumine (Sigma) in Cellular PBS buffer pH 7,4.
- Working reagent: 50:1 (v/v) BCA Reagent A: BCA Reagent B (Thermo Scientific)
- Albumine Standard Ampule, 2 mg/mL (Thermo Scientific).

12.2. Electrophoresis

Total protein was fractionated by using a 4–15% precast polyacrylamide gel with a standard Tris-glycine running buffer system. A molecular weight marker Dual color was used. Electrophoresis was performed at 120 V for 90 min.

Reagents:

- 4–15% precast polyacrylamide gel (CriterionTMTGXTM, Bio-Rad Laboratories)
- Tris-glycine running buffer 0.5x pH=8.3: 0.025 M Tris-Base (Sigma), 0.195 M glycine (Sigma) and 0.02% SDS (Sigma).
- Dual Color, Plus Protein Standards (Bio-Rad)

12.3. Electroblothing

When electrophoresis finished, fractioned proteins were electrotransferred from the gel onto a 0.2 µm nitrocellulose membrane using a Trans-blot Turbo Transfer System (Bio-Rad). To achieve this, 7 transfer papers were wetted in Trans-blot Turbo Transfer buffer solution and placed on bottom of cassette electrode (anode). Then, the nitrocellulose membrane, wetted also in Trans-blot Turbo Transfer buffer solution, was placed on top of wetted stack in the cassette, and subsequently, the gel was placed on membrane. Finally, 7 transfer papers were wetted in Trans-blot Turbo Transfer buffer solution and placed on top of 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.

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).

12.4. Labeling and detection

After electroblotting, the membrane was blocked by incubation with Odyssey Blocking Buffer Solution in TBS 1x (1:1) overnight at 4°C. After blocking, the membrane was washed with 0.1% Tween 20 in TBS 1x for 60 s and then incubated with the primary antibody specific for CPT1A, total AKT or pAKT, for 60 min at room temperature on a rocking shaker. After incubation, the membrane was washed 4 times for 5 min each one with 0.1% Tween 20 in TBS 1x at room temperature and 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. Secondary antibody was prepared in the same host species that the first antibody. Incubation with the secondary antibody was performed for 30 min at room temperature, protected from light and on a rocking shaker.

For IR detection, membranes were washed 4 times for 5 min each one 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 then was scanned in Odyssey Infrared Imaging System (LI-COR Biociences). Bands were quantified using the analysis software provided (Odyssey Software V.3.0). β-Actin was used as a housekeeping protein.

Reagents:

- Odyssey Blocking Buffer Solution (LI-COR Biociences)
- TBS 10x wash buffer, pH 7.4: 500 mL of 1M Tris-Cl (Sigma), pH 7.4 and 300 mL of 5M NaCl (Panreac), filled up to 1 L 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)
- primary rabbit polyclonal anti-CPT1A antibody (Santa Cruz Biotechnology) diluted 1:1000 in TBS 1x with 0.1% BSA (Sigma), 0.1% sodium azide (Sigma) and 0.1% Tween-20 (Sigma).
- anti-total AKT antibody (Cell Signalling Technology) diluted 1:2000 in TBS 1x with 0.1% BSA (Sigma), 0.1% sodium azide (Sigma) and 0.1% Tween-20 (Sigma).
- primary mouse polyclonal anti-pAKT (Cell Signalling Technology) diluted 1:2000 in TBS 1x with 0.1% BSA (Sigma), 0.1% sodium azide (Sigma) and 0.1% Tween-20 (Sigma).
- secondary goat anti-rabbit antibody infrared (IR)-dyed-800 (LI-COR Biociences) diluted 1:25000 in Odyssey Blocking Buffer and TBS 1x mixed 1:1, with 0.1% BSA (Sigma), 0.1% sodium azide (Sigma) and 0.1% Tween-20 (Sigma).
- secondary goat anti-mouse antibody infrared (IR)-dyed-680 (LI-COR Biociences) diluted 1:25000 in Odyssey Blocking Buffer and TBS 1x mixed 1:1, with 0.1% BSA (Sigma), 0.1% sodium azide (Sigma) and 0.1% Tween-20 (Sigma).

12.5. Scanning and quantification

The membrane was scanned on Odyssey Scanner with Infrared Imaging System (LI-COR Biosciences) in the appropriate channels IR-dyed (700 nm green channel when anti-mouse secondary antibody was used or 800 nm red channel when anti-rabbit secondary antibody was used). The quantification of all sample bands was performed using the Odyssey software V3.0 (LI-COR Biosciences). Individual features (squares) that surrounded all the fluorescent bands in the image were drawn. Then, the image data (including raw intensity, average intensity and integrated intensity) within the feature were quantified automatically. For more reliable results in the quantification the option for Integrated Intensity [the sum of the intensity values for all pixels enclosed by a feature, multiplied by the area of the feature (mm^2)] was chosen. Since background pixels should not be part of this calculation, background was calculated (Lane Background method) and subtracted.

12.6. Loading control

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

In order to stain with β -actin antibody, the membrane was incubated with mouse monoclonal anti- β -actin antibody, diluted 1:4000 (Cell Signaling). Staining with β -actin was performed according to the protocol described above in the section 12.4 for labelling and detection. The membrane was scanned, β -actin bands were quantified (according to the section 12.5 Scanning and quantification), and then, the band intensity for the specific protein was normalized to that of β -actin.

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

Reagents:

- Amido black staining solution (Sigma)

13. Indirect gas calorimetry and locomotive activity measurements

Energy expenditure and locomotive activity of our experimental rat animals was measured by 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 experiments that uses measurements of the animal's oxygen consumption (VO_2) and carbon dioxide production (VCO_2) to estimate energy expenditure. Moreover CaloSys displays a TSE InfraMot system that is designed to register total activity of one 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, rat animals were monitored for 24 h using the CaloSys. In order to reduce potential stress of the animals, they were individually housed and acclimated to the respiratory cages for 24 h before starting

measurements. After acclimation, data on gas exchanges (VO_2 , $\text{ml kg}^{-1} \text{h}^{-1}$ and VCO_2 , $\text{ml kg}^{-1} \text{h}^{-1}$) 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 8 animal cages and 1 reference cage, simultaneously). Locomotive activity (counts/h) was measured continuously by the infrared beam system of the TSE InfraMot system for 24 h.

14. Blood pressure measurement

Systolic blood pressure (SBP) was determined without anaesthesia using the tail-cuff method, a non-invasive blood pressure methodology. It consists in inflatable rubber tail-cuff sphygmomanometer with a photoelectric sensor (Niprem 546, Cibertec) placed on the animal's tail to occlude the blood flow. With this method, the arterial pulsations, which are used as endpoints for indirect determination of SBP, can be detected in the rat tail only 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 on the base of the tail for at least 30 min, to acclimate the animals and prevent hypertension by stress. During this 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 recorder continuously. Niprem software V1.8 was used to determine the SBP value and heart rate value. For each rat, SBP and heart rate values were calculated as the mean of a minimum of five measurements.

15. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS is now a routine technique with the development of electrospray ionisation (ESI), which provides a simple and robust interface. It can be applied to a wide range of biological molecules and the use of tandem mass spectrometry and stable isotope internal standards allows highly sensitive and accurate assays. Fast scanning speeds allow a high degree of multiplexing and many compounds can be measured in a single analytical run.

LC-MS/MS consists, firstly, on separating analytes and isotopic standards by ultrahigh performance liquid chromatography (UPLC) and then, the use of tandem mass spectrometry allows ionization and finally detection of the resolved analytes and isotopic standards. Mass spectrometers operate by converting the analyte molecules to a charged (ionized) state, with subsequent analysis of the ions and any fragment ions that are produced during the ionization process, on the basis of their mass to charge ratio (m/z). Several different technologies are available for both ionization and ion analysis.

During my stay in the laboratory of Professor Paul Thornalley and Dr Naila Rabani, of the Division of Metabolic and Vascular Health, Warwick Medical School (University of Warwick, Coventry, United Kingdom), I performed a quantitative screening of all the amino acids and a comprehensive range of protein damage biomarkers, in the ultrafiltrate of maternal plasma and milk samples, by using LC-MS/MS. Particularly, UPLC was performed to separate analytes and isotopic standards and then, through the mass spectrometer, the resolved analytes were detected and quantified, by using ESI as source of ionization and Quadrupole Analysers for ion analysis.

15.1. Sample preparation

Ultrafiltrates from plasma samples were prepared by microspin ultrafiltration, using a 10 KDa cut-off filter, of 60 μ l of plasma at 14000 x g for 30 min at 4°C. Ultrafiltrates from milk samples were prepared by microspin ultrafiltration, using a 3 KDa cut-off filter, of 50 μ l of milk at 14000 x g for 2 h at 4°C. UPLC vials were prepared by adding 5 μ l of plasma ultrafiltrate or 5 μ l of milk ultrafiltrate with 20 μ l of 0.1% Trifluoroacetic acid (TFA) in water and 25 μ l of a cocktail of stable isotope substituted internal standards.

15.2. Ultrahigh Performance Liquid Chromatography (UPLC)

Chromatography was performed on a graphitic stationary phase, using two 5 μ m particle size HypercarbTM columns: first column, 2.1 x 50 mm and second column, 2.1 x 250 mm (Thermo Hypersil Ltd). The chromatographic procedure was customized to complete data collection of all analytes in a 35-minute run. The mobile phase was solvent A (0.1% TFA in water) and solvent B [0.1% TFA in 50% acetonitrile (ACN)]. The two columns were initially used in series with a flow rate of 0.2 mL/min and later (at 20 min) the second column was then switched out of the flow to facilitate elution of strongly hydrophobic analytes. The post-run method for washing used solvent A1 (0.1% TFA in water) and solvent B2 [0.1% TFA in 50% tetrahydrofuran (THF)] for 30 minutes. Optimized custom gradients were used for analysis and post-run column wash/re-equilibration runs. Flow from the column in the interval 4-35 min was directed to the mass spectrometry (MS/MS) detector.

15.3. MS/MS detection

Each analyte was detected by electrospray positive ionization mass spectrometric multiple reaction monitoring (MRM). The ionization source of temperature was 150°C and the desolvation gas temperature 500°C. The cone gas and desolvation gas flow were 150 L/h and 1000 L/h, respectively. The capillary voltage and source offset were 0.50 kV and 50°C, respectively. Each analyte was detected by selective ion monitoring of its molecular ion in the first mass analyser after fragmentation with argon atoms in the collision cell. Each analyte, therefore, was detected by characteristic chromatographic retention time, molecular ion mass/charge ratio m/z and fragmentation ion m/z. Programmed molecular ion and fragment ion masses and collision energies were optimized to ± 0.1 Da and ± 1 eV, respectively, for MRM detection of analytes. Detector response was normalized to the added stable isotopic standard response, which has the same retention time as the normal analyte and a different molecular ion or molecular ion and fragment ion m/z value. Data were collected sequentially and rapidly for many mass transitions (up to 32) such that multiple mass chromatograms may be recorded for each analyte and isotopic standard.

15.4. Calculation

Analyte content was determined from the analyte/internal standard peak response peak area ratio by reference to calibration curves recorded under the same conditions with the same content of internal standard as the samples and known amounts of normal reference standard analyte. The Software MassLynx V4.1 from Waters was used to integrate analyte/internal standard peaks.

Reagents:

- Trifluoroacetic acid (TFA) (Panreac)
- Acetonitrile (ACN) (Panreac)
- Tetrahydrofuran (THF) (Panreac)

16. Statistical analysis

The methods used for statistical analyses were chosen based on the experimental questions involved. To study individual differences between experimental groups, multiple comparisons were assessed by repeated measures, one- and two-way analysis of variance (ANOVA). In some cases, Bonferroni correction was applied to prevent data from incorrectly appearing to be statistically significant by lowering the alpha value. The relationship between random variables was estimated with use of Person's correlation coefficient. In some cases, Bonferroni post hoc test was performed. Single comparisons between groups were assessed by Student's t test and Paired t test. Threshold of significance was set at $p < 0.05$. When Bonferroni correction was applied, alpha value was divided by the number of tests ($p\text{-value} = \alpha/n$). All the analyses were performed with SPSS for Windows (SPSS version 19.0, Chicago, IL).

Chapter IV.

Results and discussion

Manuscript



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

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

J Nutr Biochem. 2012;23(12):1627-39

Title page

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

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Running title: Prenatal programming of insulin/leptin resistance

Statement of financial support: The research leading to these results was supported by the Spanish Government (grant AGL2009-11277) and the European Union's Seventh Framework Programme FP7 2007-2013 under grant agreement nu 244995 (BIOCLAIMS Project). The authors' Laboratory is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, EU Contract: nu FP6-506360). The CIBER de Fisiopatología de la obesidad y nutrición is an initiative of the ISCIII.

Conflict of interest: None

Abstract

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

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

Introduction

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

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

The hypothalamus is the main organ responsible for the central control of energy balance and appetite behavior, by the production of many neuropeptides and the establishment of sympathetic connections, responding to different stimuli, such as the circulating hormones insulin and leptin (Schwartz and Porte 2005; Schwartz, et al. 2000); the brain is particularly sensitive during the perinatal period and it has been described to undergo alterations in response to particular nutritional conditions during fetal development and neonatal life (McMillen, et al. 2005). Leptin has been shown to play an important role during the perinatal period (Palou and Pico 2009). In fact, supplementation to neonate rats with physiological doses of leptin during lactation has been described to improve body weight control and leptin and insulin sensitivity in adult life (Pico, et al. 2007; Sánchez, et al. 2008), while a lack of leptin during this period occurring in leptin-deficient mice disrupts the normal postnatal developmental pattern of neural projection in the hypothalamus (Bouret, et al. 2004). Previous studies have shown that maternal 20% caloric restriction during the first half of pregnancy resulted in lower cellularity and neuropeptide Y (NPY) - and α -melanocyte-stimulating hormone (α MSH)-neurons in the arcuate nucleus (ACR) in the offspring (Garcia, et al. 2010). Delahaye et al. (Delahaye, et al. 2008) also described that maternal severe (50%) caloric restriction during both gestation and lactation reduced fiber projections from ARC neurons to other hypothalamic structures and decreased proopiomelanocortin (*Pomc*) and α *Msh* mRNA

expression levels in neonate rats. In addition, adult offspring from 30% caloric restricted pregnant dams have been shown to have the hypothalamic gene expression of *Pomc*, *Npy*, Agouti-related protein (AgRP) and leptin receptor (ObRb) altered (Ikenasio-Thorpe, et al. 2007). In addition, without neglecting the important role of the hypothalamus in regulating energy homeostasis, both white adipose tissue (WAT) and liver are also key organs in the regulation of energy balance and substrate metabolism and targets of the peripheral actions of insulin and leptin (Palou, et al. 2008). In fact, the response of these tissues to feeding conditions may be another major factor determining the higher susceptibility to developing obesity and related metabolic alterations (Palou, et al. 2008; Priego, et al. 2008).

Thus the aim of the present study was to determine the effects of moderate (20%) maternal caloric restriction during the first 12 days of gestation on determinants of later leptin and insulin resistance, by exploring the expression of selected genes involved in insulin and leptin signaling in key tissues such as the hypothalamus, rWAT and the liver, both at a juvenile age (25 days) and in adulthood (6 months, after a 2-month-period of HF diet exposure), to analyze whether this dietary stressor is able to step up early programmed disorders. Ultimately, considering the important role of leptin during a critical window of development, it was also the aim of this study to ascertain whether the effects of caloric restriction during gestation on later leptin and insulin homeostasis could be related, in part, with an alteration or a deficiency in leptin during the suckling period.

Materials and methods

Animals and experimental design

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

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

On day 25 of life, another set of control and CRG animals (n= 5-7 animals/group) (from 6 and 8 dams, respectively) were killed by decapitation under fed conditions, during the first 2 h at

the beginning of the light cycle. Blood samples were collected in heparinized containers, then centrifuged at 700 g for 10 min to obtain the plasma, and stored at -20°C until analysis. The hypothalamus, the retroperitoneal WAT (rWAT) depot and the liver were rapidly removed, weighed, frozen in liquid nitrogen and stored at -80°C until ulterior studies.

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

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

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

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

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

Blood glucose concentration was measured using Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Plasma insulin concentration was determined using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Merckodia AB, Uppsala, Sweden) following standard procedures. Plasma leptin concentration was measured using a mouse leptin ELISA kit (R&D Systems, Minneapolis, MN). Circulating triglycerides (TG) were measured by commercial enzymatic colorimetric kit (Triglyceride (INT), Sigma Diagnostics, St Louis, MO, USA).

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

RNA extraction

Total RNA was extracted from the hypothalamus, rWAT and the liver by Tripure Reagent (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop

Technologies Inc., Wilmington, Delaware, USA) and its integrity confirmed using agarose gel electrophoresis.

Real-time quantitative PCR (RT-qPCR) analysis

Real-time polymerase chain reaction (PCR) was used to measure mRNA expression levels of long form leptin receptor (*Obrb*) and insulin receptor (*Insr*) in hypothalamus, rWAT and liver of 25-day and 6 month-old rats; neuropeptide Y (*Npy*) and proopiomelanocortin (*Pomc*) in hypothalamus, tumor necrosis factor alpha (*Tnfa*), adipose triglyceride lipase (*Atgl*) and carnitine palmitoyltransferase 1 (*Cpt1*) in rWAT, and sterol response element binding protein 1c (*Srebp1c*), acetyl-coenzyme A carboxylase alpha (*Acc1*) and glycerol-3-phosphate acyltransferase (*Gpat*) in liver, in rats of 6 months of age. 0.25 µg of total RNA (in a final volume of 5 µl) was denatured at 65 °C for 10 min and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20°C for 15 min, 42°C for 30 min, with a final step of 5 min at 95°C in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain). Each PCR was performed from diluted cDNA template, forward and reverse primers (1 µM each), and Power SYBER Green PCR Master Mix (Applied Biosystems, CA, USA). Primers were obtained from Sigma (Madrid, Spain) and sequences are described in Table 1. Real time PCR was performed using the Applied Biosystems StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 min at 95°C, followed by a total of 40 two- temperature cycles (15 s at 95°C and 1 min at 60°C). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.0) and the relative expression of each mRNA was calculated as a percentage of NF control rats under *ad libitum* feeding conditions, using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). *Beta-actin* and *Gdi1* were used as reference genes depending on the tissue, age and sex of the animals, according to their better suitability.

Table 1. Nucleotide sequences of primers used for PCR amplification

Gene	Fordward Primer (5' to 3')	Reverse Primer (5' to 3')	Amplicon size (pb)
<i>β-actin</i>	GAAGCTGTGCTATGTTGCC	GGATTCCATACCCAGGAAGG	184
<i>Gdi1</i>	CCGCACAAGGCAAATACATC	GACTCTCTGAACCGTCATCAA	210
<i>Insr</i>	GTCCGGCGTTCATCAGAG	CTCCTGGGATTCATGCTGTT	242
<i>Obrb</i>	AGCCAAACAAAAGCACCATT	TCCTGAGCCATCCAGTCTCT	174
<i>Leptin</i>	TTCACACACGCAGTCGGTAT	AGGTCTCGCAGGTTCTCCAG	186
<i>Npy</i>	TGGACTGACCCCTCGCTCTAT	GTGTCTCAGGGCTGGATCTC	188
<i>Pomc</i>	CCTGTGAAGGTGTACCCCAATGTC	CACGTTCTTGATGATGGCGTTC	266
<i>Tnfa</i>	CCGATTTGCCATTTTCATACC	TCGCTTACAGAGCAATGAC	230
<i>Atgl</i>	TGTGGCCTCATTCCCTCCTAC	AGCCCTGTTTGCACATCTCT	271
<i>Cpt1</i>	GCAAACCTGGACCGAGAAGAG	CCTTGAAGAAGCGACCTTTG	180
<i>Srebp1c</i>	AGCCATGGATTGCACATTTG	GGTACATCTTTACAGCAGTG	260
<i>Acc1</i>	TGCAGGTATCCCCACTCTTC	TTCTGATTCCCTTCCCTCCT	212
<i>Gpat</i>	CAGCGTGATTGCTACCTGAA	CTCTCCGTCCTGGTGAGAAG	194

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

Western blot analysis

The amount of total insulin receptor substrate 1 (IRS1), phosphorylated IRS1 on Tyr612 (pIRS1), protein kinase C zeta (PKC ζ), signal transducer and activator of transcription 3 (STAT3) and tyrosine 705 phosphorylated STAT3 (pSTAT3) in rWAT and liver of control and CRG rats at the age of 6 months were determined by Western blot. Tissue was homogenized at 4 °C in 1:3 (w:v) or 1:20 (w:v), for rWAT and liver respectively, of lysis buffer as previously described in (Mercader, et al. 2006). The homogenate was centrifuged at 500 g for 10 min at 4 °C and the supernatant was used for protein analysis. Total protein content was measured by the method of Bradford (Bradford 1976). For analysis, 300 μ g (for rWAT analysis) or 30 μ g (for liver analysis) of total protein was solubilized and boiled for 3 min in Laemmli sample buffer containing 5% 2-beta-mercaptoethanol. Then, total protein was fractionated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE; 7.5 % polyacrylamide) and electrotransferred onto a nitrocellulose membrane (Bio-Rad, Madrid, Spain). Black amide B10 staining provided visual evidence for correct loading and blotting of proteins. After blocking, the membrane was incubated with the primary rabbit polyclonal anti-IRS1, anti-pIRS1(Tyr632), anti-PKC ζ , anti-STAT3 or anti-pSTAT3(Tyr705) antibody (Santa Cruz Biotechnology, Inc., CA, USA), and then with the infrared (IR)-dyed secondary anti-IgG antibody (LI-COR Biociences, Nebraska, USA) diluted 1:10000. For IR detection membranes were scanned in Odyssey Infrared Imaging System (LI-COR Biociences, Nebraska, USA), and the bands were quantified using the analysis software provided.

Statistical analysis

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

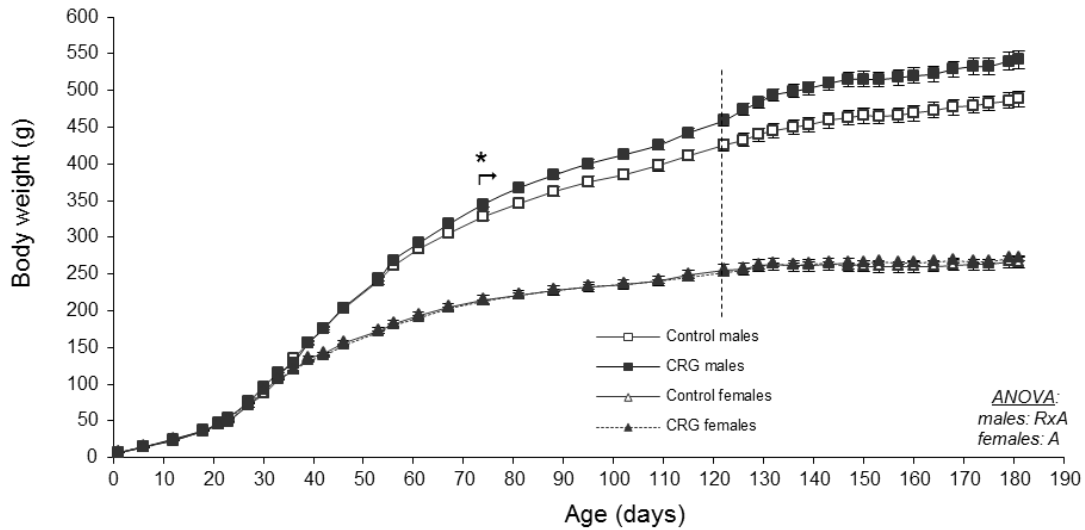
Results

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

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

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

A. Body weight evolution



B. Cumulative food intake

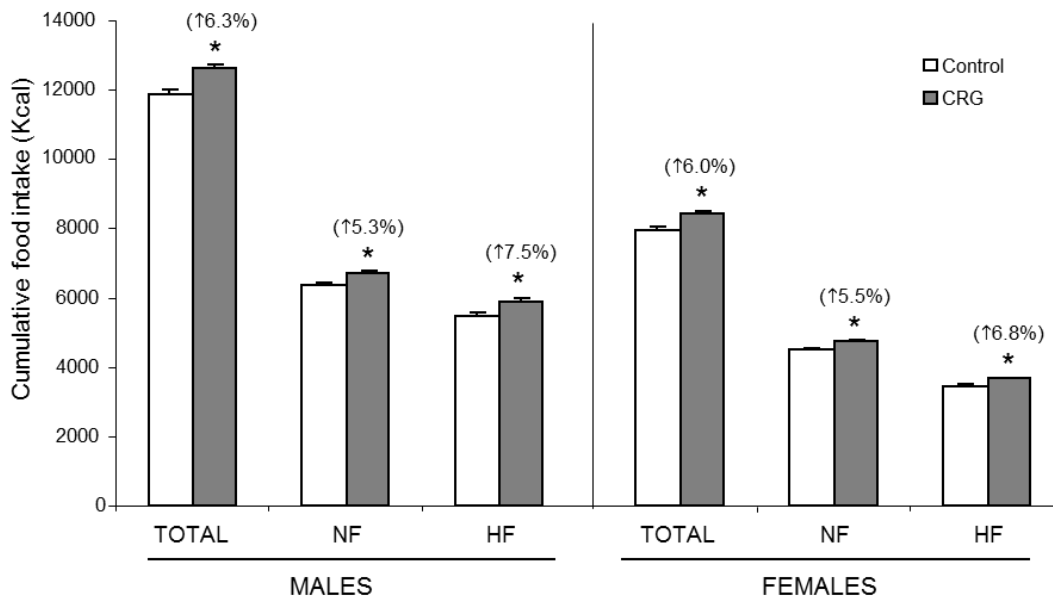


Figure 1. (A) Body weight over time until the age of 6 months of male and female offspring of controls and caloric restricted dams during gestation (CRG). Animals were fed with standard normal fat (NF) diet until the age of 4 months and then exposed to a high fat (HF) diet (the dotted line indicates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

Circulating leptin levels in control and CRG pups during lactation

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

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

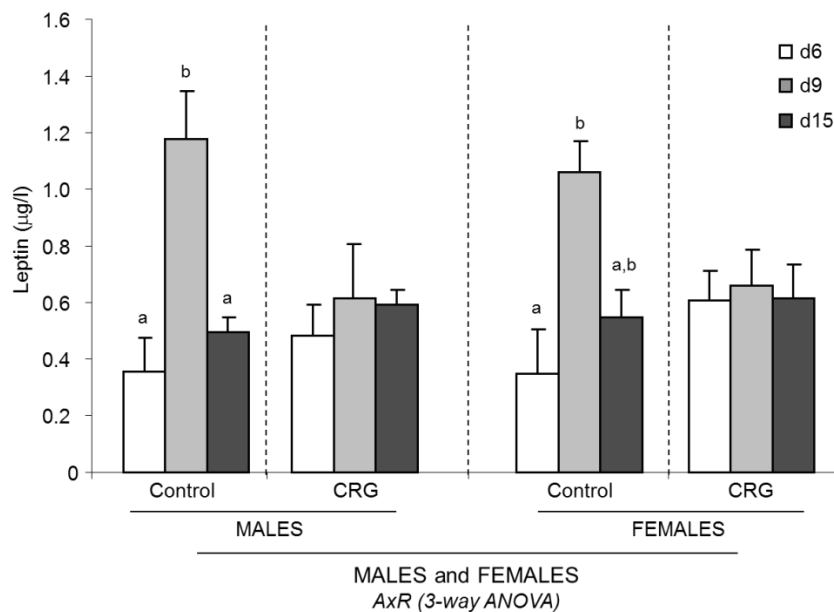


Figure 2. Plasma leptin levels ($\mu\text{g/l}$) of male and female offspring of controls and caloric restricted dams during gestation (CRG) at different stages of lactation: 6 days (d6), 9 days (d9) and 15 days (d15) of life. Data are means \pm SEM ($n = 5$ to 10). Statistics: $R \times A$, interaction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

Results in 25-day-old control and CRG rats

Body weight, tissue weights, and blood parameters

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

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

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

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

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

Gene expression levels in hypothalamus, retroperitoneal WAT and liver

Figure 3 shows mRNA expression levels of *Insr* and *Obrb* in the hypothalamus, rWAT and liver of control and CRG animals at the age of 25 days. Interestingly, both male and female CRG animals displayed lower *Insr* and *Obrb* mRNA expression levels in the hypothalamus compared with their controls (two-way ANOVA). In rWAT and liver, *Insr* mRNA levels were also significantly lower in CRG animals compared with their controls (two-way ANOVA), but no significant differences were found concerning *Obrb* mRNA levels.

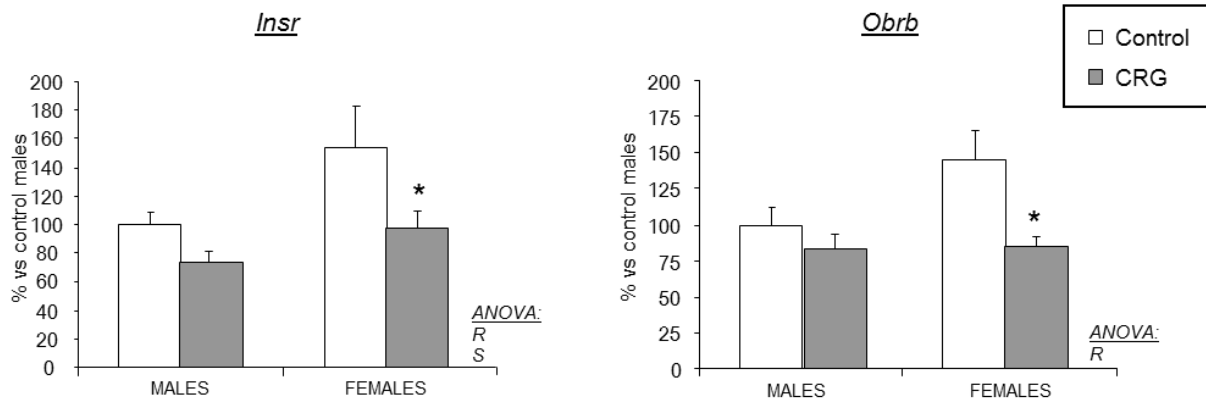
Results in 6-month-old control and CRG rats

Body weight, tissue weights and blood parameters under fed and fasting conditions

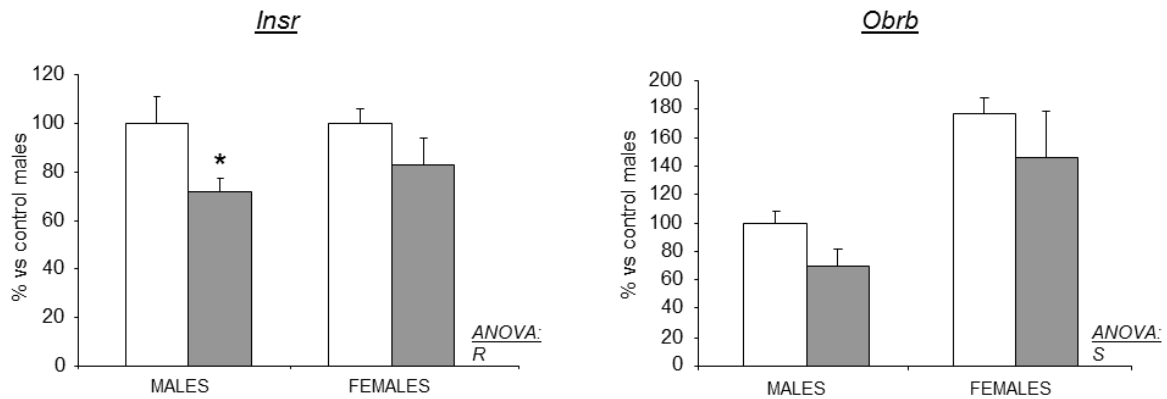
As described above, moderate maternal caloric restriction during gestation resulted in higher body weight in adulthood of male offspring but not of females (two-way ANOVA). 12-h fasting induced a significant decrease of body weight in all animals (ANOVA repeated measures) (Table 3).

The differences in body weight between control and CRG male rats can be attributed to the size of fat depots (Table 3). The adiposity index was higher in CRG males with respect to their controls (two-way ANOVA). In fact, CRG male animals showed greater fat pad weights in the 4 depots weighed, in comparison to controls (two-way ANOVA). Although no significant differences were found between control and CRG female animals, a tendency to higher adiposity was found in the latter (p=0.066, two-way ANOVA) especially due to the significantly greater gonadal fat depot (two-way ANOVA). In both males and females, no significant differences were observed by the effect of 12-h fasting in the weights of the fat depots studied, and even an increase in the size of rWAT was observed in the group of female control animals after 12 h fasting. This difference does not seem to be a direct effect of fasting, but may probably be attributed to differences in the initial weight of fat depots between animals, which could also be masking other significant effects due to the fasting state. On the other hand, gestational caloric restriction resulted in higher liver weight in CRG male animals but not in females (two-way ANOVA), especially under fasting conditions (Student's t test). As expected, fasted rats from the different groups showed lower liver weight than fed rats (two-way ANOVA).

A. Hypothalamus



B. rWAT



C. Liver

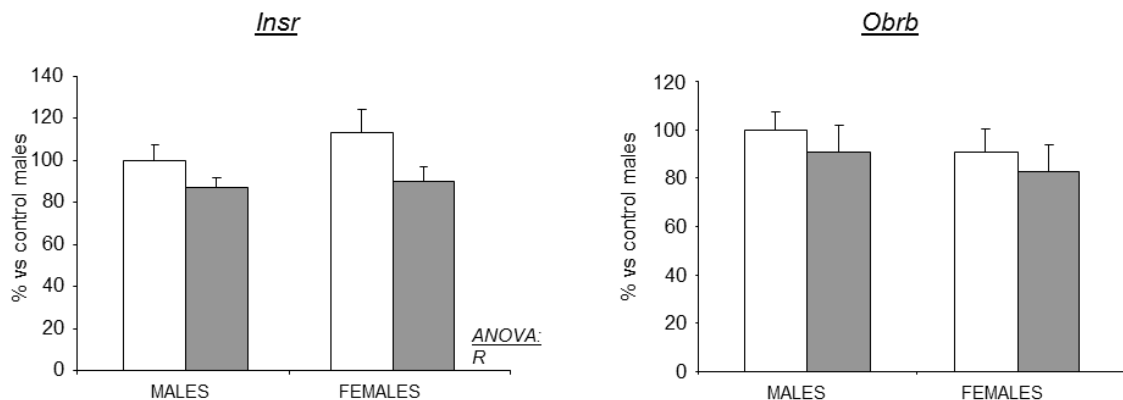


Figure 3. *Insr* and *Obrb* mRNA expression levels in hypothalamus (A), rWAT (B) and liver (C) of 25-day-old male and female offspring of controls and caloric restricted dams during gestation (CRG). mRNA levels were measured by Real-time PCR and expressed as a percentage of the value of control male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

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

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

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

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

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

Regarding leptin (Table 4), CRG male animals displayed higher circulating leptin levels than controls (two-way ANOVA), especially under fasting conditions (Student's t test). In contrast, no differences were found between control and CRG females (two-way ANOVA). As expected, circulating leptin levels decreased after 12-h fasting in male and female control and

CRG rats (two-way ANOVA).

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

Table 4. Circulating parameters at the age of 6 months

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

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

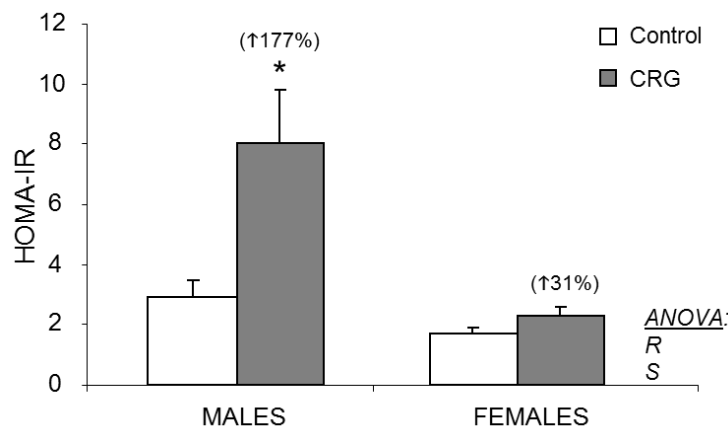


Figure 4. HOMA-IR index at the age of 6 months of male and female offspring of controls and caloric restricted dams during gestation (CRG). Results are expressed as the mean ± SEM of 6-8 animals per group. The percentage increase in HOMA-IR index of CRG compared with controls is indicated in brackets.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

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

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

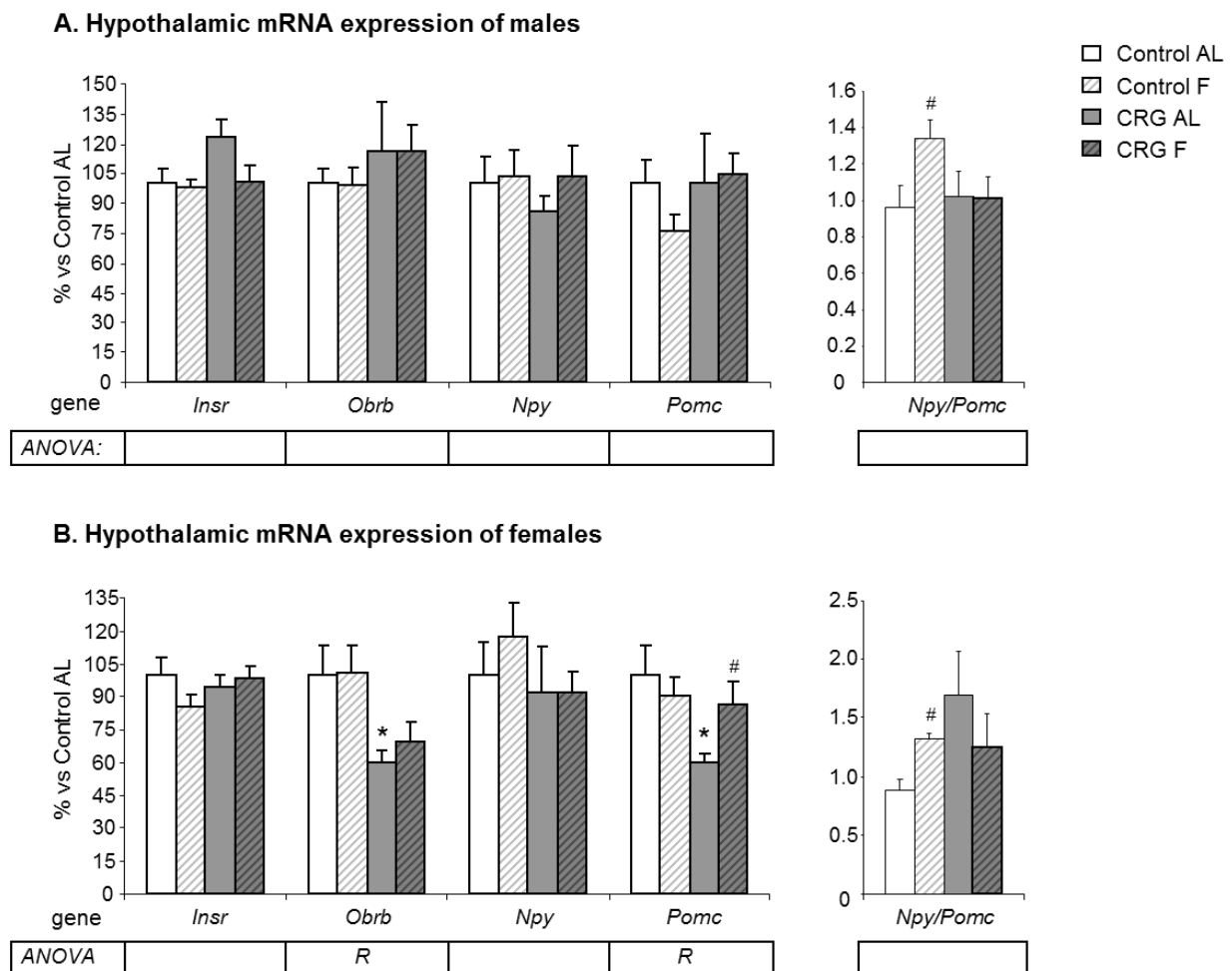


Figure 5. mRNA expression levels of insulin receptor (*Insr*), long form leptin receptor (*Obrb*), neuropeptide Y (*Npy*) and proopiomelanocortin (*Pomc*), and the *Npy/Pomc* ratio in the hypothalamus of male (A) and female (B) offspring of controls and caloric restricted dams during gestation (CRG),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

Figure 6 shows mRNA expression levels of selected genes related with energy balance in rWAT of control and CRG male and female rats. Although different adipose tissue depots were harvested, gene expression analyses were performed in the retroperitoneal depot, based on the literature showing that this depot seems to be more sensitive to nutritional status, compared with other depots (Palou, et al. 2010c). CRG male animals showed lower *Insr* and *Obrb* mRNA levels than their controls (two-way ANOVA) (Figure 6A). In addition, concerning *Insr*, their mRNA levels increased in control animals under fasting conditions, but did not change in CRG animals as an effect of food deprivation. CRG animals also showed higher *Tnfa* mRNA expression levels under fasting conditions in comparison to fasted control rats (Student's t test). Moreover CRG male animals showed a different response to fasting conditions concerning *Cpt1* expression levels: they increased in control animals under fasting conditions, but not in CRG animals. In addition, CRG male animals showed lower *Cpt1* mRNA levels under fasting conditions than their controls (Student's t test). Figure 6B shows gene expression in rWAT of female animals. Interestingly, CRG female animals, under fed conditions, showed lower *Obrb* mRNA levels than their controls. CRG animals also showed higher *Tnfa* expression levels compared to controls (two-way ANOVA). In addition there was a different response to fasting conditions between control and CRG animals concerning *Atgl* mRNA expression (interaction between caloric restriction and fasting, two-way ANOVA), since mRNA levels increased in control animals under fasting conditions, but levels did not change in CRG animals. In fact, CRG female animals under fasting conditions showed lower *Atgl* mRNA levels than their controls (Student's t test). No significant differences were found between control and CRG female animals concerning *Insr* mRNA levels.

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

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

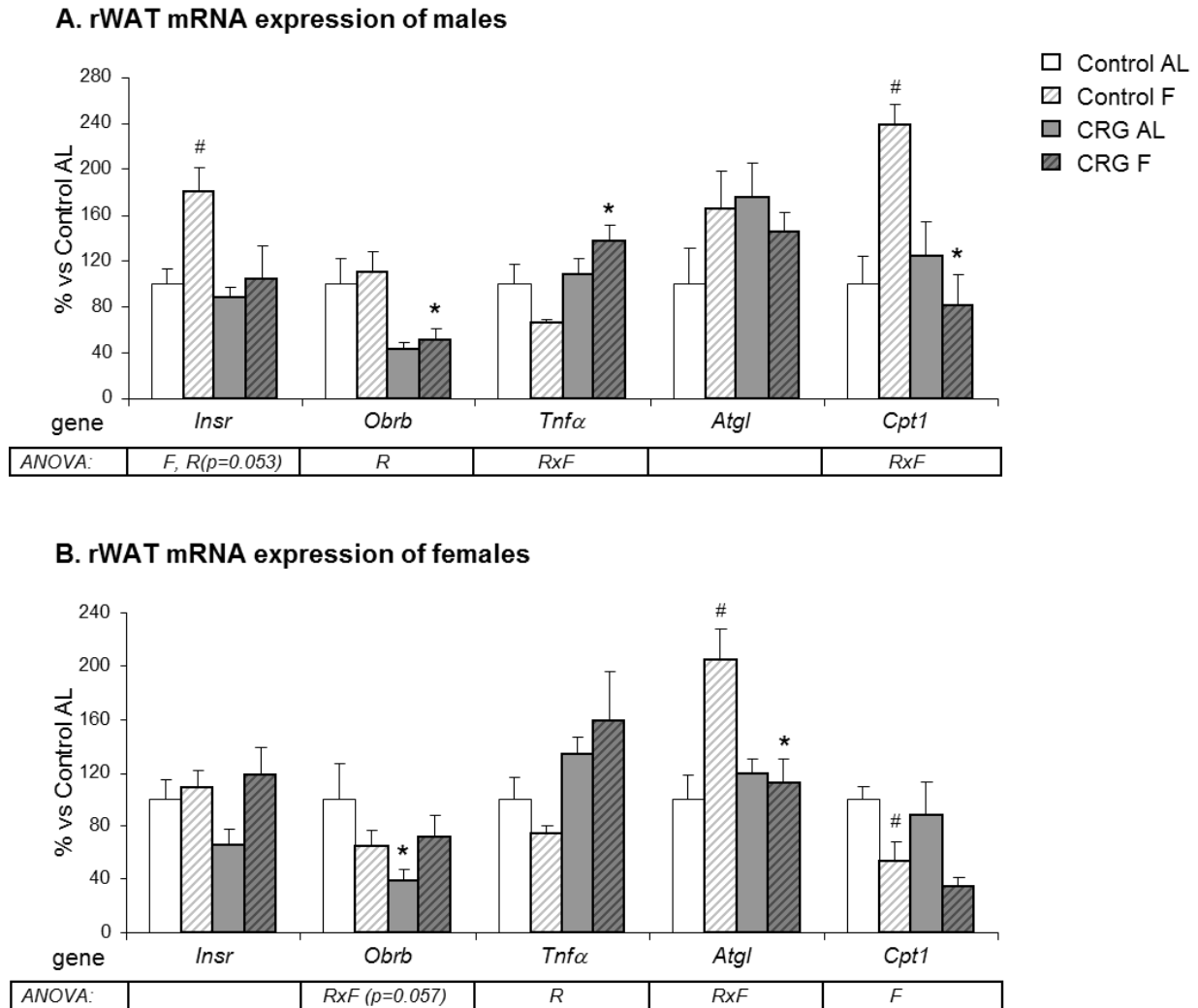


Figure 6. mRNA expression levels of insulin receptor (*Insr*), long form leptin receptor (*Obrb*), tumor necrosis factor alpha (*Tnfα*), adipose triglyceride lipase (*Atgl*) and carnitine palmitoyltransferase 1 (*Cpt1*) in rWAT of male (A) and female (B) offspring of controls and caloric restricted dams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

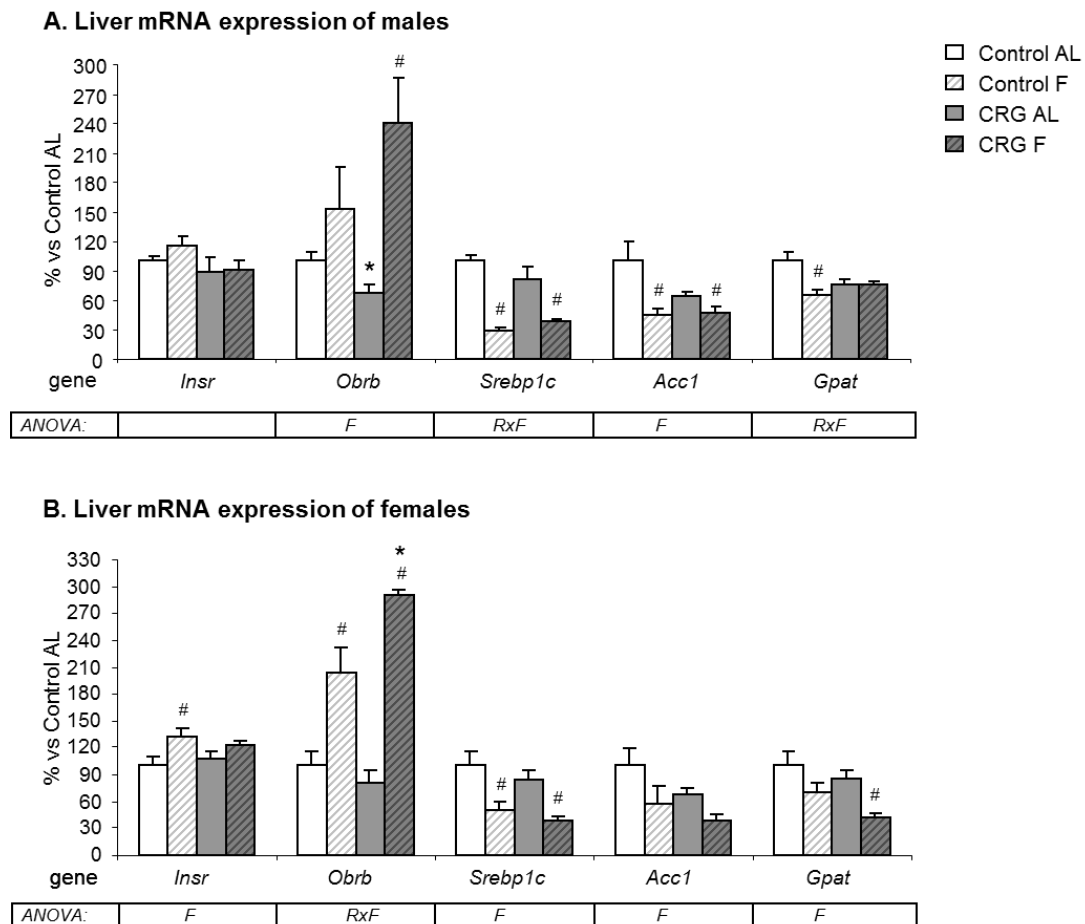


Figure 7. mRNA expression levels of insulin receptor (*Insr*), long form leptin receptor (*Obrb*), sterol response element binding protein 1c (*Srebp1c*), acetyl-coenzyme A carboxylase alpha (*Acc1*) and glycerol-3-phosphate acyltransferase (*Gpat*) in liver of male (A) and female (B) offspring of controls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

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

Table 5 shows protein levels of IRS1, pIRS1, PKC ζ , STAT3 and pSTAT3 in rWAT and liver of control and CRG animals, under fed and fasting conditions. Compared to controls, CRG male animals showed higher levels of total STAT3 in rWAT and lower PKC ζ in liver. CRG female animals showed lower levels of PKC ζ in rWAT, and lower STAT3 (only under fed conditions) and pSTAT3 in liver. No changes were found concerning IRS1 and pIRS1 between controls and CRG animals either in rWAT or liver. Fasting reduced PKC ζ and total STAT3 protein levels in rWAT of both controls and CRG male rats (two-way ANOVA), while no changes were found in female animals as an effect of fasting, with the exception of a decrease in PKC ζ levels in CRG rats (Student's t test). In liver, fasting conditions resulted in lower pIRS1 (which was higher and significant by Student's t test in controls), PKC ζ , STAT3 and pSTAT3 in males but, notably, it resulted in increased levels of IRS1, pIRS1 and of STAT3 in females (two-way ANOVA).

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

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

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

Discussion

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

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

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

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

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

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

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

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

decrease in the expression of *Obrb* has been described to be involved in the development of leptin resistance (Baskin, et al. 1998). Therefore, central insulin and leptin resistance appear to be early malprogramming effects of moderate caloric restriction during gestation, which may be responsible for impaired food intake control and hence hyperphagia in both male and female CRG animals.

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

Levels of proteins involved in insulin and leptin signaling in adult animals have been measured as they may provide evidence of the presumed altered sensitivity to these hormones. Results obtained for PKC are supportive as CRG female animals showed lower levels of PKC δ compared with their controls. This decrease was more marked under fasting conditions. PKC δ is a downstream effector in the insulin signaling pathway and plays an important role in activating the glucose transport response; in fact, overexpression of PKC δ or constitutively active PKC δ has an insulin-like effect on glucose transport during *in vitro* incubation of different kinds of cell lines (rev.(Liu, et al. 2006)). Thus, the decrease in PKC δ protein levels in rWAT of CRG females suggests a decrease in insulin signaling and glucose uptake by the adipose tissue. Decreased PKC δ levels in insulin target organs, such as muscle, have also been found in different experimental models of undernutrition during gestation and related with major propensity to insulin resistance development in adulthood (Chen, et al. 2009).

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

Even though changes in proteins involved in insulin and leptin signaling in adult CRG animals under HF diet did not prove a decreased adipose tissue sensitivity to these hormones, compared with controls, it is interesting to highlight that both male and female CRG rats showed higher expression levels of the proinflammatory cytokine $Tnf\alpha$. $Tnf\alpha$ is overexpressed in adipose tissue in many rodent models of obesity and affects insulin sensitivity (Hotamisligil, et al. 1993). In fact a link between obesity, insulin resistance and inflammation has been proposed (Gual, et al. 2005). $TNF\alpha$ has been involved in the JNK mediated serine phosphorylation of IRS1, which inhibits the normal tyrosine phosphorylation of IRS1 in response to insulin (Gual, et al. 2005). In adult CRG males, obesity was clearly manifested, but CRG females did not present overweight at that moment of life, but these results, in accordance with other alterations in the expression of other key genes in WAT, such as the *Obrb* gene, suggest a dysregulation of energy homeostasis in these animals. Since females have been described to be more protected against the detrimental effects associated to obesity compared to males (Priego, et al. 2008) and CRG females presented severe alterations in other factors involved in the control of energy balance and appetite behavior at the central and peripheral level, it could be thought that CRG females might be in a previous stage of the disease and could develop excessive fat accumulation, together with other endocrine alterations, later on in life. In favor of the detrimental programming effects of maternal caloric restriction during gestation in the female offspring is the lack of increase in *Atgl* mRNA expression levels in rWAT under fasting conditions, which occurs in control animals, in agreement with the literature (Palou, et al. 2008); this could be indicative of an impaired capacity to mobilize TG from this tissue under a negative energy balance situation. On the other hand, and in accordance with their higher fat accumulation in the adipose tissue, CRG male animals did not exhibit an increase in *Cpt1* mRNA levels under fasting conditions, while this increase was found in control animals.

Regarding the liver, at the age of 25 days, CRG rats presented lower *Insr* mRNA levels and no significant changes in *Obrb* mRNA levels compared to controls. Interestingly, when adult, and under HF diet, CRG males showed lower *Obrb* mRNA levels than controls, but only under fed conditions, with no changes in *Insr* mRNA levels. Nevertheless, CRG males also showed lower PKC δ protein levels than their controls. In addition, although hepatic levels of IRS1 and STAT3 did not change between control and CRG animals, it must be noted that their phosphorylated levels (Tyr 632 and Tyr 705, respectively) showed a diminished response to fasting conditions compared with their controls. All in all, these results agree with impaired leptin and insulin signaling in liver, which could be associated with the impaired response to changes in fed/fasting conditions concerning lipogenesis. In fact, CRG males displayed a lower or even a lack of response to fasting conditions concerning the expression levels of *Srebp1c* and *Gpat*, respectively. Unlike males, no changes were found in females between control and CRG animals concerning the expression of insulin and leptin receptors, or of other genes related with lipid metabolism. CRG females even showed higher *Obrb* mRNA levels than their controls under fasting conditions. This does not seem to be indicative of higher leptin signaling in these animals, since they displayed lower protein levels of total STAT3 (only under fed conditions) and of their phosphorylated (Tyr705) form, which suggests lower leptin signaling. The function of leptin in liver is not clearly established, but it has been shown to have antisteatotic effects by lowering the expression of SREBP1. In fact, mice with ablated hepatic leptin signaling have increased lipid accumulation in the liver (Kakuma, et al. 2000). Thus, leptin appears to act as a negative regulator of insulin action in liver (Huynh, et al. 2010), and therefore impaired leptin action in liver may affect whole energy homeostasis.

Thus, these results concerning the expression levels of insulin and leptin receptors in peripheral tissues such as WAT and liver at early stages of life, together with the apparent impaired action of these hormones found in adult life, suggests that early programming of peripheral insulin

resistance may be a direct consequence of fetal caloric restriction, while peripheral leptin resistance, which appears in adulthood and under HF diet, might be secondary to insulin resistance or to central leptin resistance. In fact it is recognized that elevated insulin levels promote both insulin resistance and increased leptin biosynthesis and secretion from adipose tissue, which may further desensitize leptin signaling and increase leptin resistance (Seufert 2004). However, although the leptin-obesity-insulin resistance link is established, which alterations are causes or consequences in this particular situation and why the phenotypic outcomes are different between males and females needs further clarification.

All in all, we show here that maternal caloric restriction during gestation results in early effects on the expression of leptin and insulin receptors in key tissues involved in energy homeostasis, particularly hypothalamus, which may compromise the proper functioning of the leptin and insulin systems. Moderate caloric restriction has also been associated with malprogramming of central hypothalamic structures involved in energy balance (Garcia, et al. 2010) and with a reduction in adipose tissue sympathetic innervation (Garcia, et al. 2011). However, the mechanisms or factors responsible for the detrimental effects of these perinatal conditions are not clearly established. Leptin, which is naturally present in maternal milk, is known to play a key role during the suckling period (Palou and Pico 2009). Leptin supplementation (physiological doses) in rats during the suckling period has later effects in the offspring preventing overweight in adulthood (Pico, et al. 2007) as well as other alterations related with the metabolic syndrome (Priego, et al. 2010), and also improves later insulin and leptin sensitivity (Sánchez, et al. 2008). These beneficial effects of leptin during lactation seem to be related, at least in part, with a better control of food intake, associated with epigenetic changes in the promoter methylation of *Pomc* (Palou, et al. 2011). Moreover, in rodents, it is known that plasma leptin levels rise transiently during neonatal period, peaking around day 10 of lactation, a process that has been termed as “neonatal leptin surge” (Ahima, et al. 1998; Pico, et al. 2011 May 4; Rayner, et al. 1997). This surge in leptin levels seems to be important for programming the structural and functional development of hypothalamic orexigenic and anorexigenic centers (Grove and Smith 2003) and its potential alteration by maternal caloric restriction has been checked here. In control animals we observed the expected peak in circulating leptin levels at this period (9 days of life), but it is suggestive that this was absent in CRG animals. A premature or delay in leptin peak, as occurring in mice with severe fetal undernutrition (Yura, et al. 2005) or in protein restricted rats during gestation and during both gestation and lactation (Zambrano, et al. 2006), has also been associated with obesity in adulthood. Nevertheless, whether the malprogramming effects observed by caloric restriction could be simply attributed to the lack of the leptin surge needs to be specifically addressed. In any case, these results underscore the importance of leptin during lactation and the critical consequences that leptin deficiency may have during a critical period in postnatal life, being responsible for the common, detrimental outcomes of different adverse perinatal conditions.

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

Manuscript

2

Enhancing hepatic fatty acid oxidation
as a strategy for reversing metabolic
disorders programmed by maternal
undernutrition during gestation

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Cell Physiol Biochem. 2014;33(5):1498-515

Title page

Title: Enhancing hepatic fatty acid oxidation as a strategy for reversing metabolic disorders programmed by maternal undernutrition during gestation

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Running title: Metabolic malprogramming reversion by increasing FAO

Statement of financial support: We gratefully acknowledge the Spanish Government (grant AGL2012-33692 to A.P., grant SAF2010-20039 to L.H. and grant SAF2011-30520-C02-01 to D.S.), the European Union (BIOCLAIMS FP7-244995 to A.P.), the Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red Fisiopatología de la Obesidad y Nutrición (CIBEROBN), EFSD/Lilly and EFSD/Janssen (research fellowships to LH) and by L'Oréal-UNESCO (research fellowship to LH). The Laboratory of Molecular Biology, Nutrition and Biotechnology (Nutrigenomics) is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, EU Contract: n° FP6-506360).

Conflict of interest: None

Abstract

Background/Aims: Moderate maternal calorie-restriction during gestation programmes offspring for a major propensity to develop metabolic alterations in adulthood. We aimed to assess whether increased hepatic fatty-acid oxidation (FAO), at early ages, by gene transfer of *Cpt1am* (active mutant of carnitine palmitoyltransferase-1a), may be a strategy for reversing metabolic disturbances associated to maternal calorie-restriction during gestation in rats.

Methods: AAV-*Gfp* (control) and AAV-*Cpt1am* vectors were administered by tail vein injection in 18-day-old control-pups and the offspring of 20% calorie-restricted rats during gestation (CRG). After weaning, animals were fed with normal-fat diet. At the age of 4 months, they were moved to HF-diet and sacrificed at the age of 6 months to collect tissues. Locomotive activity, energy expenditure and blood pressure were measured.

Results: Under HF-diet, CRG-animals showed higher HOMA-IR, adipocyte diameter and hepatic triglyceride accumulation than controls; these alterations were reverted in *Cpt1am*-injected animals. In liver, this treatment ameliorated inflammatory state, decreased expression of lipogenesis-related genes and partially restored the decreased expression of leptin-receptor occurring in CRG-animals. Treatment also reverted the decreased energy expenditure and the increased blood pressure of CRG-animals.

Conclusion: Increasing hepatic FAO through AAV-*Cpt1am* injection at juvenile ages prevents some metabolic disorders associated to gestational maternal calorie-restriction.

Keywords: *Cpt1a*, calorie restriction, gestation, gene transference, liver

Introduction

Obesity prevalence is increasing worldwide and hence the risk for related chronic diseases such as insulin resistance, type 2 diabetes, dyslipidemia, cardiovascular diseases, hypertension and nonalcoholic fatty liver disease (NAFLD) (Malik, et al. 2013). Besides the contribution of genetic and environmental factors, growing evidence links nutritional and other environmental conditions during early life to the subsequent development of obesity and other features of the metabolic syndrome (Cottrell and Ozanne 2008; Taylor and Poston 2007). Development of new strategies to prevent or treat obesity and its related metabolic disorders by reverting metabolic programming may be of interest.

Gestation is a critical period of development, and food restriction during this stage has been described to induce permanent metabolic changes in the offspring increasing the propensity to obesity and related risk parameters in adult life (McMillen, et al. 2008). In this regard, we have previously shown that moderate (20%) calorie restriction in rats during the first half of gestation programmes the offspring for a lower capacity to respond to insulin and to central leptin action and results in higher food intake in adulthood (Palou, et al. 2012; Palou, et al. 2010a). Male offspring show higher and earlier harmful effects than females, while females appear more resistant to the detrimental effects of gestational calorie restriction, in terms of maintenance of body weight (Palou, et al. 2012a).

Bearing in mind that the ultimate cause of obesity is an imbalance between energy intake and expenditure, strategies enabling the energy balance to be inclined towards fatty-acid oxidation (FAO) could improve obesity-related disorders. In fact, chronic increased hepatic FAO through adeno-associated virus (AAV)-mediated gene transfer of the key gene for mitochondrial β -oxidation, carnitine palmitoyltransferase 1a (*Cpt1a*), or to a better extent *Cpt1am*, a permanently active form of CPT1A insensitive to its physiological inhibitor malonyl-CoA, have been shown to reduce high-fat (HF) diet-induced hepatic steatosis, weight gain, inflammation, diabetes and insulin resistance in mice (Orellana-Gavaldà, et al. 2011). Genetically obese *db/db* mice expressing *Cpt1am* also show lower glucose and insulin levels and reduced liver steatosis (Orellana-Gavaldà, et al. 2011). Short-term studies that increase FAO in liver by genetic transfer of *Cpt1a* also show a decrease in hepatic triglyceride (TG) content in obese rats (Stefanovic-Racic, et al. 2008). Therefore, increased hepatic FAO in adult animals by overexpression of *Cpt1a* has been proposed as a new potential therapeutic target against obesity-induced disorders (Orellana-Gavaldà, et al. 2011; Stefanovic-Racic, et al. 2008).

Considering that offspring of 20% calorie restricted dams is an animal model with a major propensity to suffer obesity, hyperinsulinemia and leptin resistance (Palou, et al. 2012; Palou, et al. 2010a), here we aimed to assess whether increased hepatic FAO at early ages of life through AAV-mediated gene transfer of *Cpt1am* may be a strategy to trigger a persistent reversion of metabolic disturbances related to developmental malprogramming.

Methods and Materials

Adeno-associated vectors

AAV vectors serotype 1, AAV1-AAT-*Gfp* and AAV1-AAT-*Cpt1am*, driving mouse liver expression of green fluorescence protein (*Gfp*) and *Cpt1am*, respectively, were used. Details of these constructs are described in (Orellana-Gavaldà, et al. 2011). Briefly, vector plasmids carried human albumin enhancer element, human 1-antitrypsin (EalbAATp) liver-specific promoter, the cDNA sequence of *Gfp* and *Cpt1am*, woodchuck posttranscriptional regulatory element (WPRE, Access. No.AY468-486) and bovine growth hormone polyadenosine transcription termination signal [bGHPoly(A)] (bases 2326-2533 GenBank Access. No.M57764). The expression cassette was flanked by two inverted terminal repeats (ITRs) derived from AAV2. AAV1 vectors were produced in insect cells using baculovirus. The vector preparations used had titers of 1×10^{12} and 7.5×10^{11} genome copies (gc)/ml for AAV1-AAT-*Gfp* and AAV1-AAT-*Cpt1am*, respectively.

Animals and Experimental Design

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

The study was conducted on male *Wistar* rats from 16 different litters following the protocol as is described below. The study was performed with male animals because they have been shown to be more sensitive to the effects of maternal undernutrition during gestation compared to females, concerning features of the metabolic syndrome (Palou, et al. 2012; Palou, et al. 2010a).

Animals were housed under standard conditions, that is, controlled temperature (22°C), a 12h light-dark cycle and free access to tap water. 16 virgin female *Wistar* rats weighing between 220g and 250g 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 either control (n= 7 dams) or calorie restricted (n= 9 dams) groups. Control dams had free access to standard chow diet (3kcal/g, with 8% calories from fat; Panlab, Barcelona, Spain), while calorie restricted (CRG) dams underwent 20% restriction of calorie intake from day 1 to day 12 of pregnancy. Calorie restriction was performed by offering each dam a daily amount of food corresponding to 80% of the calories they should be eaten according to body weight. This amount was calculated by 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*. At day 1 after delivery, pups were weighed and the size of all litters was adjusted to 10 neonates per dam (five males and five females, when possible). During the lactating period, control and CRG dams had unlimited access to standard chow diet. Body weight and food intake of both control and CRG dams were measured throughout the whole periods of gestation and lactation.

At the age of 18 days, AAV1 vectors were administered by tail vein injection in control (n= 21) and CRG (n= 19) male pups in a single dose of 7.5×10^{12} genome copies/kg of body weight, as described (Orellana-Gavaldà, et al. 2011). Before treatment, pups were anesthetized with isofluorane. Half of the male control and CRG pups were injected with the control construct

(AAV-*Gfp*), and referred to as Control-C and CRG-C, respectively. The other half of pups were injected with AAV1 vectors carrying the cDNA for *Cpt1am* (AAV-*Cpt1am*), and referred to as Control-CPT and CRG-CPT, respectively. After injections, pups were returned to their respective dams.

All pups were weaned at 21 days of life, and then housed one per cage and fed on a normal-fat (NF) standard chow diet (3.8kcal/g, with 10% calories from fat; Research Diets, NJ, ScholarOne, 375 Greenbrier Drive, Charlottesville, VA, 22901 USA) until the age of 4 months. Then, all animals were exposed to HF diet (4.7kcal/g, with 45% calories from fat; Research Diets, Inc., NJ, USA) until the age of 6 months. HF diet contained 5.5% calories from soybean oil and 39.5% from lard. Body weight and food intake of the offspring were recorded from weaning until the age of 6 months, when animals were killed. Locomotive activity and energy expenditure were measured at the age of 3 months by indirect calorimetry. Blood pressure was also measured at the age of 5 months. Two weeks before sacrifice, blood samples of all animals were collected after 12h fasting from the saphenous vein. Plasma was obtained as described above to analyze circulating parameters. Finally, at the age of 6 months, the four groups of animals were killed under *ad libitum* feeding conditions by decapitation during the first 2h at the beginning of the light cycle and on different consecutive days. At killing, the liver and retroperitoneal white adipose tissue (rWAT) were rapidly removed and weighed. A part of the two tissues was immediately frozen in liquid nitrogen and stored at -80°C until analysis. Another part of the liver was immediately homogenised to isolate mitochondria. Finally, pieces of liver and rWAT were collected for histological analysis. To avoid adipocyte and hepatocyte damage, and to preserve the integrity of their respective membranes, liver and rWAT samples were immediately fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer (pH= 7.4) at 4°C for 24h. Then, fixed samples were washed and stored in 0.1M phosphate buffer (pH= 7.4) until subsequent analysis. Trunk blood samples were also collected in heparinized containers. Plasma was obtained by centrifugation of heparinized blood at 1000 x g for 10min and stored at -20°C until analysis of circulating parameters.

GFP detection in hepatocytes

The presence of GFP was examined in hepatocytes of animals injected with the AAV-*Gfp* vector by using a confocal laser scanning microscope. To prepare samples, pieces of frozen liver were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer (pH= 7.4) at 4°C for 24h, and washed in 0.1M phosphate buffer (pH= 7.4) for 24h. Then, samples were immersed in 30% sucrose in PBS buffer 1x for 72h at 4°C. Before sectioning, the microtome chamber of the cryostat (Microtome cryostat HM 505 E, Microm International GmbH, Walldorf, Germany) was set at -25°C (optimal cutting temperature for liver) for 24h. Finally, tissues were embedded in O.C.TTM compound (Sakura Finetek, The Netherlands) on the specimen stage and put into the fast freezing station for 24h. Fifteen micrometer-thick sections of tissues were cut and mounted on slides. Liver sections were washed in PBS buffer 1x and hepatocyte nuclei were stained with blue-fluorescence DAPI dilactate 300nM in PBS buffer (Life Technologies S.A., Madrid, Spain) according to the manufacturer's instructions. Finally, slides were mounted with Dako Fluorescence Mounting Medium (Dako Diagnostics, S.A. Barcelona, Spain), cover-slipped and protected from light. Microscopic examinations were conducted with an automated inverted microscope DMI 4000B coupled with Leica TCS SPE Confocal Laser System (Leica Microsystems S.L.U., Barcelona, Spain). The fluorescence spectral properties selected were: for DAPI, 405nm excitation maximum and emission spectral range from 418 to 468nm; for GFP, 488nm excitation maximum and emission spectral range from 500 to 550nm. Digital images were acquired by scanning with the fluorescence spectral

properties described above and the projections of the individual channels were merged in Leica Application Suite Software 2.3.6 to facilitate visualization.

Isolation of mitochondria from liver

Mitochondria from liver samples were isolated as previously described (Orellana-Gavaldà, et al. 2011) with minor modifications. Briefly, 2g of liver obtained from 6-month-old animals at sacrifice were immediately homogenized in 20mL of a buffer solution (70mM sucrose, 220mM mannitol, 2mM EDTA, 5mM HEPES and protease inhibitor cocktail from Roche, pH= 7.4) by using a glass homogenizer Potter-Elvehjem, and centrifuged at 600 x g for 15min at 4°C. The supernatant was centrifuged again at 12000 x g for 20min at 4°C. The pellet was resuspended in 2mL of buffer solution and centrifuged at 7000 x g for 10min at 4°C. Finally, the mitochondrial-enriched pellet was resuspended in 1mL of buffer solution and stored at -80°C until used for Western blot analysis.

Western blot analysis

Western blot was performed to determine CPT1A, total AKT (serine/threonine protein kinase) and phosphorylated AKT on Serine 473 (pAKT) in homogenates of the whole liver and of CPT1A in homogenates of the hepatic mitochondrial fraction of the animals at the age of 6 months.

Liver was homogenized at 4°C in 1:10 (w:v) of RIPA lysis buffer 1x (1M Tris HCl buffer pH=7.4, containing 1M NaCl, 10% deoxycholic acid, 10% NP40 and 0.5M EDTA) with protease and phosphatase inhibitor cocktail (Halt™ Protease & Phosphatase Inhibitor Cocktail 100x, Thermo Fisher Scientific Inc, Madrid, Spain). The homogenate was centrifuged at 7500 x g for 2min at 4°C, and the supernatant was used for protein analysis. Total hepatic protein content was measured with the Pierce BCA protein assay kit (Thermo Scientific, Rockford, U.S.A). For analysis, 60µg (for CPT1A) or 50µg (for total AKT and pAKT) of total protein was solubilized and boiled for 3min in Laemmli sample buffer containing 5% 2-β-mercaptoethanol. Then, total protein was fractionated by using a 4–15% precast polyacrylamide gel (Criterion™TGX™, Bio-Rad Laboratories, Inc, U.S.A) with a standard Tris-glycine running buffer system and subsequently electrotransferred onto a 0.2µm nitrocellulose membrane (Bio-Rad, Madrid, Spain). To electrotransfer, a Trans-blot Turbo Transfer System was used, following the manufacture's instructions (Bio-Rad, Madrid, Spain). After blocking, the membrane was incubated with primary rabbit polyclonal anti-CPT1A antibody (Santa Cruz Biotechnology, Inc., CA, USA) diluted 1:1000 or anti-total AKT antibody (Cell Signalling Technology, Inc., Danvers, MA) diluted 1:2000, and then with secondary goat anti-rabbit antibody infrared (IR)-dyed-800 (LI-COR Biociences, Nebraska, USA) diluted 1:25000. For pAKT analysis, the membrane was incubated with primary mouse polyclonal anti-pAKT (Cell Signalling Technology, Inc., Danvers, MA) diluted 1:2000, and then with secondary goat anti-mouse antibody infrared (IR)-dyed-680 (LI-COR Biociences, Nebraska, USA) diluted 1:25000. For IR detection, membranes were scanned in Odyssey Infrared Imaging System (LI-COR Biociences, NE, USA), and the bands were quantified using the analysis software provided (Odyssey Software V.3.0). β-Actin was used as a housekeeping protein.

CPT1A was also determined in the hepatic mitochondrial fraction. Mitochondria were precipitated from the mitochondrial-enriched pellet in buffer solution by centrifuging at 7000 x g for 10min at 4°C. The pellet was sonicated at 4°C in 150µl of cellular PBS buffer with

protease and phosphatase inhibitor cocktail (Halt™ Protease & Phosphatase Inhibitor Cocktail 100x, Thermo Fisher Scientific Inc, Madrid, Spain). The homogenate was centrifuged at 7500 x g for 2min at 4°C, and the supernatant was used for protein analysis. Total mitochondrial protein content was measured by the method of Bradford (Bradford 1976). For analysis, 40µg of total mitochondrial protein was solubilized and boiled for 3min in Laemmli sample buffer containing 5% 2-β-mercaptoethanol. Western blot and CPT1A detection was performed as described above.

Histological analysis

rWAT and liver samples from all groups were processed. Firstly, fixed samples were dehydrated in graded series of ethanol, cleared in xylene and embedded in paraffin blocks. Then, five micrometre-thick sections of tissues were cut with a microtome and mounted on slides.

For histological analysis, slides were stained with hematoxylin and eosin, dehydrated with ethanol, cleared in xylene and mounted with Eukitt (Panreac Quimica SA). For each rWAT and liver sample, 2-3 photos from the most preserved and representative sections of the slides were digitalized from light microscopy (Zeiss Axioskop 2 microscope) connected with AxioCam Icc3 digital camera (Carl Zeiss, S.A., Barcelona, Spain). In liver sections, the presence of fatty vesicles was studied by using the software AxioVision 40V 4.6.3.0. (Carl Zeiss, Imaging Solutions GmbH, Germany). Morphometric analyses were performed in rWAT. The diameter of all adipocytes from each photo of rWAT was measured by using the software AxioVision 40V 4.6.3.0. mentioned above, and then averaged. Only adipocytes which had an intact membrane were measured. Image analyses from all samples and groups were examined in a blind fashion.

Measurement of circulating parameters

Circulating parameters were measured at the age of 6 months under fed and/or fasting conditions. Blood glucose concentration was measured by Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Commercial enzyme-linked immunosorbent assay (ELISA) kits were used for the quantification of circulating plasma levels of hormones: insulin (Mercodia AB, Uppsala, Sweden), and adiponectin, leptin and TNFα (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Absorbance of the samples was read with a spectrophotometer Tecan Sunrise Absorbance Reader. A fluorimetric assay was used for the quantification of β-hydroxybutyrate (BHB) plasma levels (kit from BEN S.r.l.-Biochemical enterprise). Fluorescence was detected by using a spectrofluorimeter Mithras LB 940 (Berthold Technologies GmGH & Co KG, Bad Wildbad, Germany).

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

Hepatic triglycerides, extraction and quantification

Lipid extraction was performed in the liver of 6-month-old animals to evaluate hepatic TG content. The protocol followed was described in Caimari *et al.* (Caimari, et al. 2012) with some

modifications. Briefly, 50-100mg of liver was mixed with 1mL of hexane/isopropanol (3:2, vol/vol). Hepatic lipid extracts were resuspended in 1.2mL of LPL buffer (28.75mM Pipes, 57.4 mM $MgCl_2 \cdot 6H_2O$, 0.569mg/mL bovine serum albumin-fatty acid free) with sodium dodecyl sulfate (SDS) 0.1%. Then, samples were sonicated for 30s, and tubes were left overnight in an orbital shaker at room temperature and protected from light. On the following day, tubes were cold sonicated with three pulses of 30s each, and their TG levels were measured immediately using a commercial enzymatic colorimetric kit (Triglyceride (INT) 20, Sigma Diagnostics, St Louis, MO, USA).

Quantification of hepatic glycogen levels

Hepatic glycogen was quantified in liver samples from 6-month-old animals. Glycogen isolation was performed by digestion of 0.3-0.8g of liver in 1 mL of 30% KOH at 100°C for 10min. Glycogen was precipitated overnight at -20°C with 2mL of 100% ethanol and collected by centrifugation at 3000rpm for 30min at 4°C. The precipitate was redissolved in 1mL of 8% trichloroacetic acid (TCA) and centrifuged at 3000rpm for 15min at 4°C; the supernatant was stored at 4°C, whereas the pellet was redissolved in 1mL of 8% TCA and centrifuged at 3000rpm for 15min at 4°C; the resulting supernatant was added to the stored one. Glycogen was again obtained from the supernatant by precipitation with 4mL of 96% cold ethanol followed by centrifugation at 3000rpm for 15min at 4°C. Finally, the pellet was then redissolved in 1mL of water. Glycogen concentration was measured with the anthrone reagent as previously described (Roe and Dailey 1966).

Indirect calorimetry and locomotive activity measurements

At the age of 3 months, animals were monitored for 24h to assess energy expenditure by indirect calorimetry and locomotive activity by using the LabMaster-CaloSys-Calorimetry System (TSE Systems, Bad Homburg, Germany). Before starting measurements, and in order to reduce potential stress, animals were individually housed and acclimated to the respiratory cages for 24h. Then, data on gas exchanges (VO_2 ; $ml\ kg^{-1}\ h^{-1}$ and VCO_2 ; $ml\ kg^{-1}\ h^{-1}$) were measured via an open-circuit indirect calorimetry system for 24h. To calculate energy expenditure (kcal/h), rates of oxygen consumption and carbon dioxide production were monitored for 5min every 45min for each animal or reference cage (our system can handle 8 animal cages and 1 reference cage, simultaneously). Locomotive activity (counts/h) was measured continuously by an infrared beam system integrated in the LabMaster System for 24h.

Blood pressure measurement

Systolic blood pressure (SBP) was measured when animals were 5 months old. SBP was determined without anaesthesia using non-invasive blood pressure methodology. It consists of using a tail-cuff sphygmomanometer with a photoelectric sensor (Niprem 546, Cibertec S.A., Spain) placed on the animal's tail to occlude the blood flow. Heart rate was also registered. Niprem software V1.8 was used to determine the SBP value and heart rate value. For each rat, SBP and heart rate values were calculated as the mean of a minimum of five measurements.

Gene expression analysis in liver

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

Real-time quantitative polymerase chain reaction (RT-qPCR) was used to measure mRNA expression levels in liver of *Acaca* (Acetyl-CoA carboxylase subunit alpha), *Cd68* (cluster of differentiation 68), *Fasn* (fatty acid synthase), *G6pd* (glucose-6-phosphate dehydrogenase), *ObRb* (long form leptin receptor) and *Tnfa* (tumor necrosis factor alpha). *Gdi1* (guanosine diphosphate (GDP) dissociation inhibitor 1) and *Rn18s* (18S ribosomal RNA) were used as housekeeping genes. All primers used for RT-qPCR amplification were obtained from Sigma Genosys (Sigma Aldrich Química SA, Madrid, Spain) and sequences are described in (Palou, et al. 2012; Palou, et al. 2009a; Ribot, et al. 2008), except for: *G6pd* (forward sequence: 5'-CGAACCACATCTCCTCTCTG-3' and reverse sequence 5'-ATCAAAATAGCCCCACGAC-3') and *Cd68* (forward sequence: 5'-AATGTGTCCTTCCCACAAGC-3' and reverse sequence 5'-GGCAGCAAGAGAGATTGGTC-3').

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

Statistical analysis

Data are mean \pm standard error of the mean (S.E.M.). Multiple comparisons were assessed by two-way or repeated measures analysis of variance (ANOVA) to determine the effects of calorie restriction during gestation, and AAV-*Cpt1am* injection vs AAV-*Gfp*. Single comparisons between groups were assessed by Student's *t* test and the effect of fasting conditions by Paired *t* test. Threshold of significance was set at $p < 0.05$, unless indicated. Analysis was performed with SPSS for Windows (SPSS version 19.0, Chicago, IL).

Results

Vector insertion verification in hepatic cells

The AAV-*Gfp* and AAV-*Cpt1a* constructs used here were the same as in a previous study (Orellana-Gavaldà, et al. 2011). Their capacity to insert in hepatic cells and drive overexpression of the target genes had been previously proven (Orellana-Gavaldà, et al. 2011).

Since vector insertion was made at an early age of life (postnatal day 18), relative *Cpt1a* expression was expected to be decreased as the animals grew. Western Blot analysis of CPT1A in liver was performed in adult animals to verify whether overexpression of *Cpt1a* remained at the age of 6 months. Fig. 1 shows protein levels of hepatic CPT1A in the whole protein extract in liver (Fig. 1a) and levels of hepatic CPT1A in the mitochondrial protein fraction in liver (Fig. 1b). When analysing the whole protein extract, we did not observe significant changes in CPT1A protein levels due to the injection of AAV-*Cpt1a* vectors (no significant differences by two-way ANOVA). Control-CPT and CRG-CPT animals tended toward slightly higher but not significantly different levels of CPT1A than those animals injected with control-vectors. When analysing the mitochondrial protein extract, Control-CPT animals showed greater CPT1A levels than Control-C rats ($p < 0.05$, Student's *t* test). No changes were observed in CPT1A protein content by effect of maternal calorie restriction during gestation (no significant differences by two-way ANOVA).

The presence of GFP in hepatocytes of animals injected with control (AAV-*Gfp*) vectors was used to demonstrate the achievement of injection and vector insertion. AAV-*Cpt1a* injected animals were used as a negative control. Relative *Gfp* expression was also expected to decrease as the animals grew, but was still detected in adult animals. Fig. 2 shows digital images of representative liver slides of Control-C, Control-CPT, CRG-C and CRG-CPT animals acquired by using confocal laser scanning microscopy. Fluorescent signals from GFP were found as spots distributed in the cytoplasm of hepatocytes of Control-C and CRG-C animals (GFP images), while no green fluorescence emission was observed in hepatocytes of Control-CPT and CRG-CPT. Nuclear DAPI staining is also shown in blue (DAPI images). Merge images show the superposition of DAPI and GFP images, evidencing the cell localisation of green fluorescence.

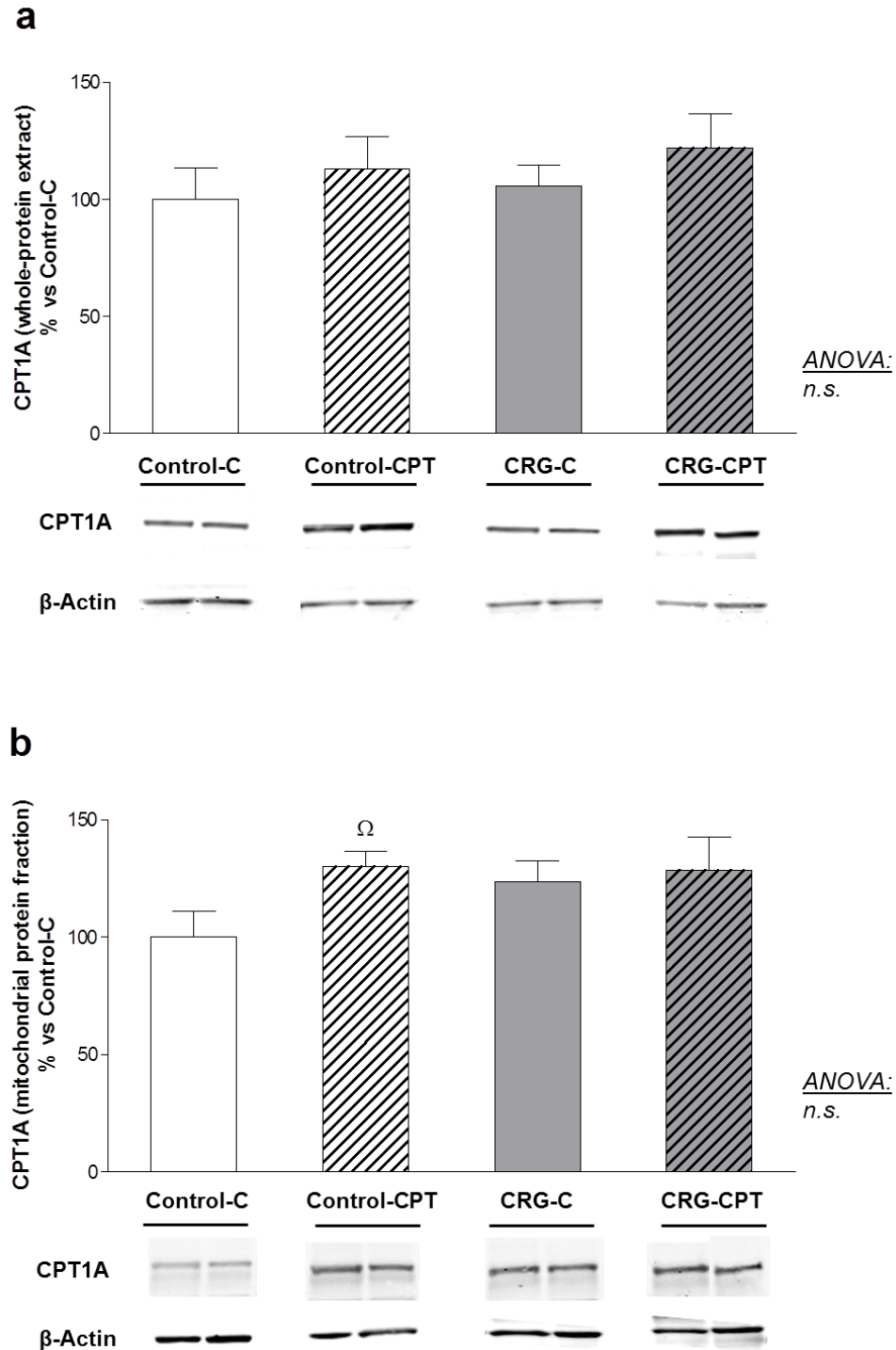


Figure 1. Protein levels of CPT1A measured in whole-protein extracts of liver (a), or in the mitochondrial protein fraction (b) from 6-month-old offspring of control dams injected on postnatal day 18 with *Gfp* and *Cpt1am* vectors (Control-C and Control-CPT, respectively) and offspring of calorie restricted dams during gestation injected with *Gfp* and *Cpt1am* vectors (CRG-C and CRG-CPT, respectively). Data are mean \pm S.E.M. (n= 9-11 animals per group). Statistics: Ω , *Cpt1am* vs *Gfp*-injection, ($p < 0.05$, Student's *t* test). Abbreviations: carnitine palmitoyltransferase 1A (CPT1A); no significant differences (n.s.).

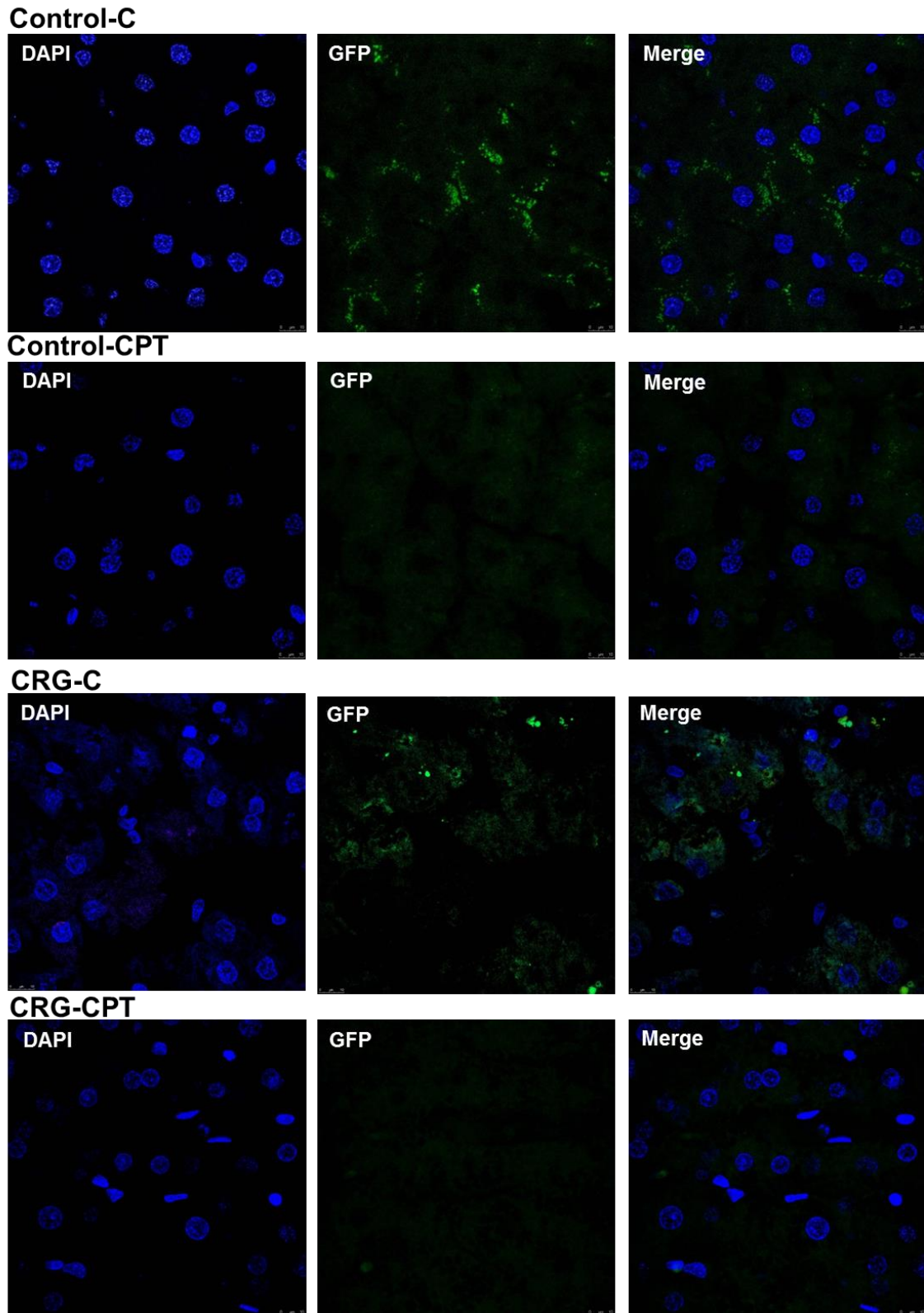


Figure 2. GFP detection in hepatocytes from 6-month-old offspring of control dams and calorie restricted dams during gestation, injected on postnatal day 18 with *Gfp* (Control-C and CRG-C, respectively) compared to those AAV-*Cpt1am* injected ones (Control-CPT and CRG-CPT, respectively). Detection was made by confocal laser scanning microscopy of frozen liver sections stained with DAPI for nucleus identification. Excitation wavelength was set at 405nm (for DAPI) and 488nm (for GFP). Images were scanned at 64X. Abbreviations: blue-fluorescence DAPI dilactate (DAPI) and green fluorescence protein (GFP).

AAV-Cpt1am treatment at early ages improved insulin sensitivity and prevented increased adiposity size occurring in the offspring of calorie restricted dams during gestation without affecting body weight

Body weight

As shown in Table 1, moderate maternal calorie restriction during the first 12 days of pregnancy did not result in different offspring body weight at birth with respect to their controls. Neither were significant differences found between control and CRG animals throughout the study period, either under NF-diet or when moved to a HF diet. Neither did AAV-Cpt1am injection affect body weight of animals compared to their AAV-Gfp injected control ones.

The weight of WAT depots (inguinal or retroperitoneal) was not different between groups at the age of 6 months.

Table 1. Body weight at different ages and weight of WAT depots and liver at sacrifice

	Age	Control		CRG	
Body weight (g)					
Birth	PND-1	6.72 ± 1.16		6.72 ± 0.20	
		Control-C	Control-CPT	CRG-C	CRG-CPT
AAV-Injection	PND-18	35.5 ± 0.8	35.8 ± 0.9	35.6 ± 1.6	34.9 ± 1.4
Weaning	PND-21	45.8 ± 1.2	46.4 ± 1.1	46.1 ± 2.4	45.0 ± 1.9
Before HF-diet	4 months	411 ± 9	401 ± 8	407 ± 9	399 ± 11
After HF-diet	6 months	479 ± 10	471 ± 13	478 ± 14	464 ± 14
WAT weight (g)					
iWAT	6 months	12.4 ± 0.8	13.2 ± 0.9	13.7 ± 1.2	11.7 ± 1.0
rWAT	6 months	17.8 ± 1.4	18.4 ± 1.4	18.9 ± 1.7	18.5 ± 1.2
Liver weight (g)					
	6 months	13.6 ± 0.4	12.8 ± 0.6	12.5 ± 0.3	12.6 ± 0.5

At birth, data are mean ± S.E.M (n=19-21 animals per group) of the offspring of control and calorie restricted dams during gestation (control and CRG, respectively). From PND-18, data are mean ± S.E.M (n=9-11 animals per groups) of the offspring of control dams injected in postnatal day 18 with *Gfp* and *Cpt1am* vectors (Control-C and Control-CPT, respectively) and the offspring of calorie restricted dams during gestation injected with *Gfp* and *Cpt1am* vectors (CRG-C and CRG-CPT, respectively). Abbreviations: inguinal white adipose tissue (iWAT), postnatal day (PND), and retroperitoneal white adipose tissue (rWAT).

Circulating parameters

Table 2 shows circulating levels of glucose, insulin, BHB, TG, leptin, adiponectin and TNFα of control (Control-C and Control-CPT) and CRG (CRG-C and CRG-CPT) animals at the age of 6 months under feeding or 12h fasting conditions, as indicated. Leptin to adiponectin (L/A) ratio and HOMA-IR are also presented. CRG animals (both CRG-C and CRG-CPT) showed higher fasted insulin and HOMA-IR than controls (p<0.05 two-way ANOVA). It is worth noting that, AAV-Cpt1am injected animals showed lower fasted insulin levels (only control animals, p<0.05, Student's *t* test) and lower HOMA-IR (both control and CRG animals, p<0.05 two-way ANOVA) than their respective AAV-Gfp controls. CRG-CPT animals also showed lower fasted glucose levels than CRG-C animals (p<0.05, Student's *t* test). CRG-CPT animals also showed a response to fasting conditions, regarding glucose and insulin levels, while this response was not shown in CRG-C animals.

Table 2. Circulating parameters at the age of 6 months

		Control-C	Control-CPT	CRG-C	CRG-CPT	ANOVA
Glucose (mg/dL)	Fed	121 ± 3	115 ± 2	111 ± 4	117 ± 4	<i>R</i> × <i>T</i>
	Fasting	104 ± 3 [#]	107 ± 4 [#] <i>p</i> =0.06	110 ± 4	97.1 ± 1.0 ^{#, Ω, *}	
Insulin (µg/L)	Fed	2.09 ± 0.18	2.84 ± 0.54	2.54 ± 0.46	3.54 ± 0.56	<i>R</i>
	Fasting	0.974 ± 0.154 [#]	0.583 ± 0.100 ^{#, Ω}	1.62 ± 0.41	1.19 ± 0.24 ^{#, *}	
BHB (µmol/L)	Fed	666 ± 84	652 ± 70	466 ± 112	596 ± 79	<i>R</i>
	Fasting	841 ± 73	911 ± 222	2000 ± 194 ^{#, *}	1433 ± 205 ^{#, Ω}	
TG (mg/mL)	Fed	2.23 ± 0.31	1.67 ± 0.28	1.57 ± 0.22	1.54 ± 0.22	
	Fasting	0.408 ± 0.042 [#]	0.446 ± 0.095 [#]	0.457 ± 0.078 [#]	0.380 ± 0.096 [#]	
Leptin (µg/L)	Fed	12.2 ± 1.2	12.4 ± 1.4	12.4 ± 1.4	11.2 ± 1.1	
	Fasting	4.93 ± 0.58 [#]	5.97 ± 0.77 [#]	7.25 ± 1.0 ^{#, *} <i>p</i> =0.062	6.76 ± 0.67 [#]	
Adiponectin (mg/L)	Fed	9.36 ± 0.82	10.4 ± 0.9	8.89 ± 0.37	9.05 ± 1.20	
	Fasting	8.76 ± 0.62	8.85 ± 0.55	8.05 ± 0.59	9.02 ± 0.50	
L/A ratio (µg/mg)	Fed	1.31 ± 0.14	1.31 ± 0.20	1.41 ± 0.15	1.42 ± 0.21	
	Fasting	0.58 ± 0.08 [#]	0.68 ± 0.08 [#]	0.92 ± 0.15 ^{#, *}	0.70 ± 0.07 [#]	
HOMA-IR	Fasting	6.25 ± 1.06	3.77 ± 0.67 ^Ω <i>p</i> =0.07	10.7 ± 2.8	6.97 ± 1.14 [*]	<i>R, T</i>
TNFα (pg/mL)	Fed	4.40 ± 0.80	4.66 ± 0.80	6.62 ± 0.77 [*] <i>p</i> =0.066	5.50 ± 1.15	

Circulating parameters under fed and fasting conditions from 6-month-old offspring of control dams injected in postnatal day 18 with *Gfp* and *Cpt1am* vectors (Control-C and Control-CPT, respectively) and offspring of calorie restricted dams during gestation injected with *Gfp* and *Cpt1am* vectors (CRG-C and CRG-CPT, respectively). Data are mean ± S.E.M (n=9-11 animals per group) Statistics: *R* × *T*, interaction between maternal calorie restriction during gestation and *Cpt1am*-injection; *R*, effect of maternal calorie restriction during gestation; *T*, effect of *Cpt1am*-injection (*p*<0.05, two-way ANOVA). #, fasting vs feeding conditions (*p*<0.05, Paired *t* test); *, CRG vs Control animals; ^Ω, *Cpt1am* vs *Gfp*-injection (*p*<0.05, Student's *t* test). Abbreviations: BHB, (β-hydroxybutyrate), HOMA-IR (insulin resistance HOMA index), L/A ratio (leptin-to-adiponectin ratio), TG (triglycerides) and TNFα (tumor necrosis factor alpha).

Concerning leptin, CRG-C animals revealed a trend to higher leptin levels under fasting conditions than controls (*p*=0.062, Student's *t* test). In addition, although adiponectin levels were not significantly different between groups, L/A ratio of fasted CRG-C animals was significantly higher compared to Control-C animals (*p*<0.05 Student's *t* test). Differences were not observed between CRG-CPT and Control-CPT animals.

Regarding BHB, under fasting conditions, CRG animals showed higher levels than their controls (*p*<0.05 two-way ANOVA); CRG-CPT animals displayed lower levels than CRG-C animals (*p*<0.05, Student's *t* test). No differences between groups were observed concerning TG levels. Finally, plasma levels of TNFα tended toward being higher in CRG-C animals compared to Control-C rats (*p*=0.066, Student's *t* test). No changes were observed between the other groups of animals.

Protein levels of total AKT and pAKT in liver

The potential effect of increased hepatic *Cpt1a* expression at early ages on insulin signalling was evaluated by studying total AKT and pAKT in liver at the age of 6 months (Fig. 3). CRG animals injected with AAV-*Cpt1am* vectors showed a lower pAKT/total AKT ratio compared to CRG-C and Control-C animals (*p*<0.05, Student's *t* test). Thus, an interaction between calorie restriction and *Cpt1am*-injection was observed for total AKT and pAKT/total AKT ratio (*p*=0.057 and 0.056, respectively, two-way ANOVA).

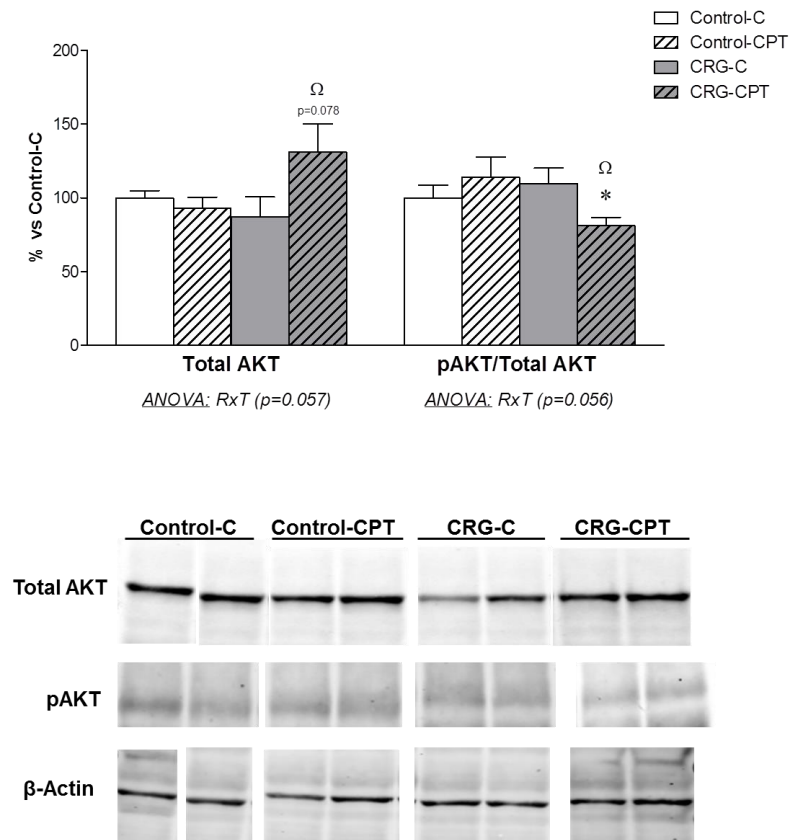


Figure 3. Protein levels of total AKT and pAKT/total AKT ratio in liver from 6-month-old offspring of control dams injected on postnatal day 18 with *Gfp* and *Cpt1am* vectors (Control-C and Control-CPT, respectively) and offspring of calorie restricted dams during gestation injected with *Gfp* and *Cpt1am* vectors (CRG-C and CRG-CPT, respectively). Data are mean \pm S.E.M. (n= 9-11 animals per group). β -Actin was used a housekeeping protein. Statistics: *RxT*, interaction between maternal calorie restriction during gestation and *Cpt1am*-injection, (two-way ANOVA). Ω , *Cpt1am* vs *Gfp*-injection); *, CRG vs Control animals (p<0.05, Student's *t* test). Abbreviations: total protein kinase B (total AKT) and phosphorylated AKT on Serine 473 (pAKT).

Composition and histological analyses of liver and rWAT

Fig. 4a shows the hepatic content of TG of animals at the age of 6 months. CRG-C animals tended toward displaying higher hepatic TG levels than their controls (p=0.081, Student's *t* test) while no differences were observed between the other groups. Fig. 4b shows representative images of hematoxylin/eosin stained liver slides of animals from the different groups. All the animals presented fatty vesicles in their hepatocytes but CRG animals showed a greater amount of fat vesicles than control animals, especially CRG-C animals. Interestingly, fewer fatty vesicles were observed in CRG-CPT animals compared to those injected with control-vectors.

Regarding hepatic glycogen content (Fig. 4c), AAV-*Cpt1am* injected animals revealed a trend to higher levels, although differences did not reach statistical significance (p=0.087, two-way ANOVA).

Fig. 5a shows the diameter of adipocytes measured in sections of rWAT from animals at the age of 6 months. Representative rWAT sections stained with hematoxylin/eosin of the different groups of animals are also given (Fig. 5b). Although no significant differences were observed concerning the weight of WAT depots, adipocytes of CRG-C animals had a larger diameter

than Control-C animals; interestingly, this effect was not seen in AAV-*Cpt1am* injected CRG animals. CRG-CPT animals presented adipocytes with a significantly lower diameter than CRG-C rats ($p < 0.05$ two-way ANOVA).

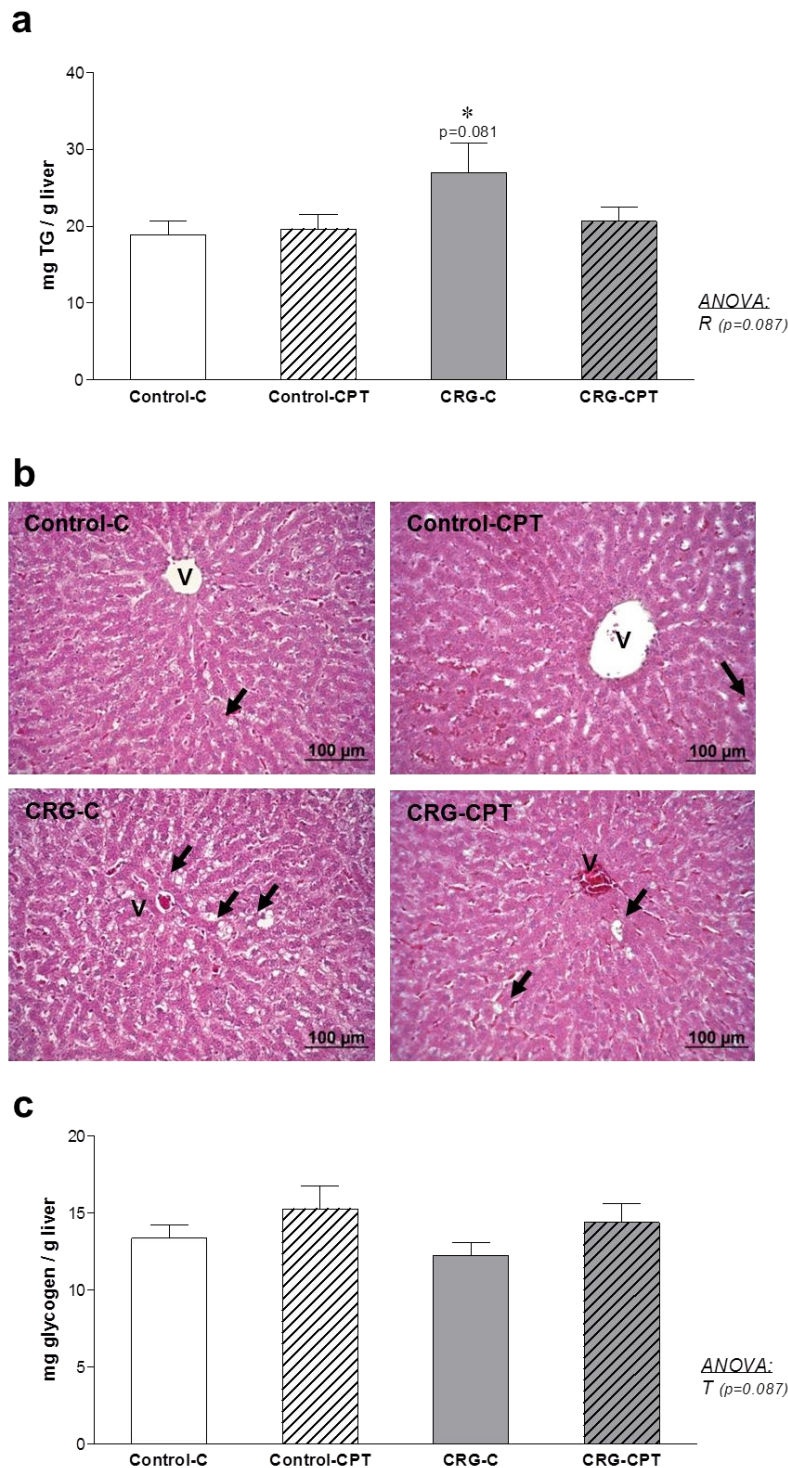


Figure 4. Triglyceride content in liver (**a**); representative liver slides stained with hematoxylin/eosin (x20); (**b**) and hepatic glycogen content (**c**) from 6-month-old offspring of control dams injected on postnatal day 18 with *Gfp* and *Cpt1am* vectors (Control-C and Control-CPT, respectively) and offspring of calorie restricted dams during gestation injected with *Gfp* and *Cpt1am* vectors (CRG-C and CRG-CPT, respectively). Data are mean \pm S.E.M. (n= 9-11 animals per group). Statistics: *R*, effects of maternal calorie restriction during gestation ($p=0.087$, two-way ANOVA); *T*, effects of *Cpt1am*-injection ($p=0.087$, two-way ANOVA). *, CRG vs Control animals, ($p < 0.05$, Student's *t* test). Abbreviations: triglycerides (TG). Symbol images: V, indicate a central vein; the arrows indicate some lipid droplets.

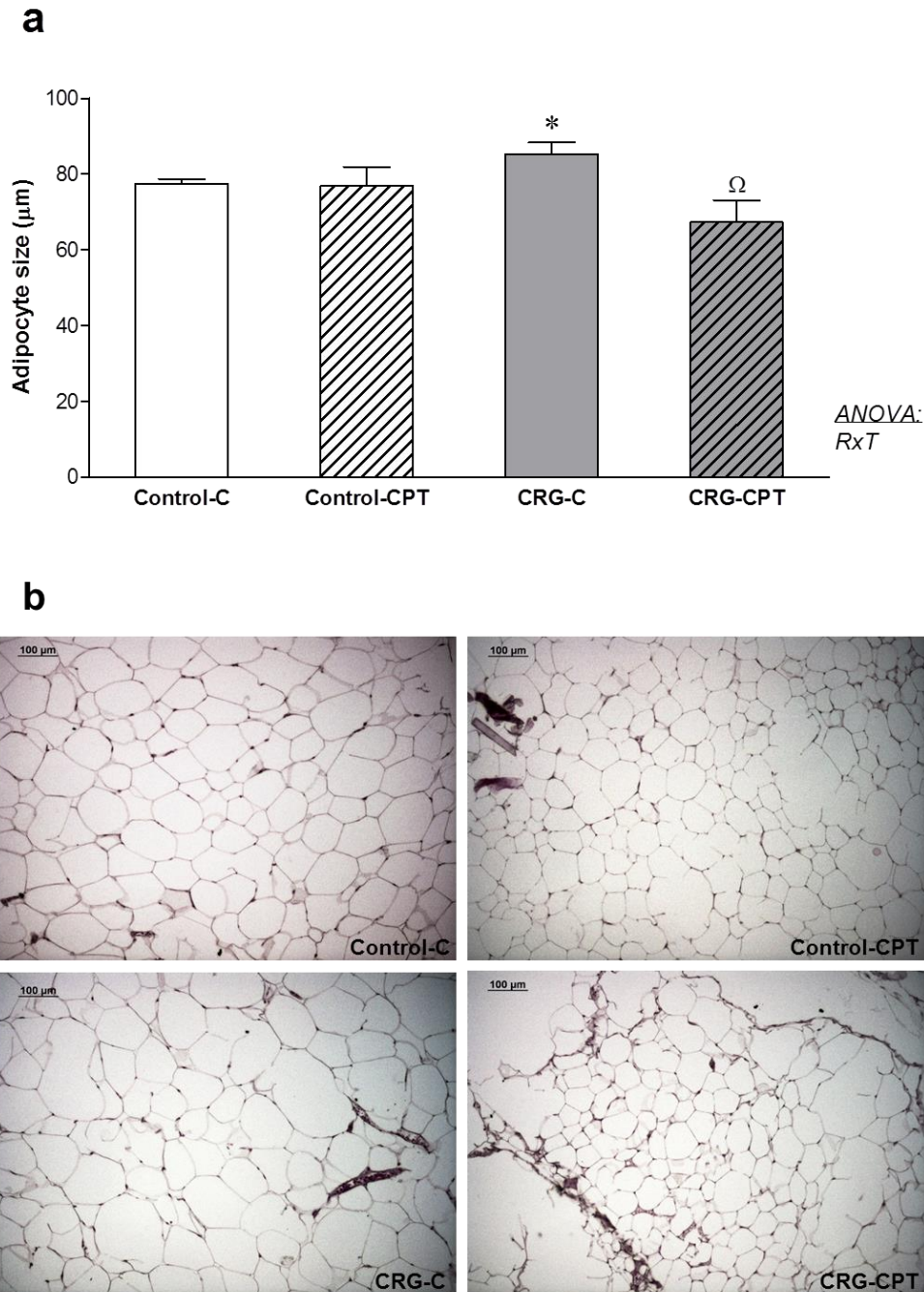


Figure 5. Adipocyte size in rWAT (**a**) and images (20x) of rWAT sections with hematoxylin/eosin staining (**b**) from 6-month-old offspring of control dams injected on postnatal day 18 with *Gfp* and *Cpt1am* vectors (Control-C and Control-CPT, respectively) and offspring of calorie restricted dams during gestation injected with *Gfp* and *Cpt1am* vectors (CRG-C and CRG-CPT, respectively). Data are mean \pm S.E.M. (n= 9-11 animals per group). Statistics: RxT, interaction between maternal calorie restriction during gestation and *Cpt1am*-injection ($p < 0.05$, two-way ANOVA). *, CRG vs Control animals; Ω , *Cpt1am* vs *Gfp*-injection ($p < 0.05$, Student's *t* test). Abbreviations: rWAT (retroperitoneal white adipose tissue).

AAV-Cpt1am treatment at early ages restored changes in energy metabolism, locomotive activity and systolic blood pressure programmed by calorie restriction during gestation

Energy expenditure

Energy expenditure was measured by indirect calorimetry at the age of 3 months. As shown in Fig. 6a, CRG animals, both CRG-C and CRG-CPT, showed lower energy expenditure than their controls ($p < 0.05$, repeated measures ANOVA) and especially during the nocturnal period ($p < 0.05$, Student's t test). Despite the lower energy expenditure observed in CRG animals, CRG-CPT rats displayed higher energy expenditure during the nocturnal period than CRG-C animals ($p < 0.05$, Student's t test). Energy expenditure was significantly higher during the nocturnal period in all groups of animals than during the diurnal period ($p < 0.05$, repeated measures ANOVA).

Locomotive activity

Locomotive activity of animals at the age of 3 months is shown in Fig. 6b. CRG-C animals showed a trend to lower locomotive activity compared to their controls; this tendency was not shown in CRG-CPT animals, which exhibited similar levels to control animals. In fact, CRG-CPT animals displayed higher activity than CRG-C animals during the diurnal period ($p < 0.05$, Student's t test), but a trend toward higher activity was also observed during the nocturnal phase ($p = 0.073$, Student's t test). Interactive effects were found between light (diurnal or nocturnal period), calorie restriction during gestation and *Cpt1am*-injection ($p < 0.05$, repeated measures ANOVA). When diurnal and nocturnal period were analysed separately, an interaction between calorie restriction and *Cpt1am*-injection was found during the nocturnal period ($p < 0.05$, two-way ANOVA). Considering all animals as a whole, locomotive activity during the nocturnal phase showed a positive correlation with energy expenditure ($r = 0.414$; $p = 0.010$, Pearson's correlation).

Systolic blood pressure

SBP was measured when animals were 5 months old (Fig. 6c). CRG-C animals displayed a significantly higher SBP than their Control-C animals ($p < 0.05$, Student's t test). Interestingly, CRG-CPT animals did not show significant differences in SBP in comparison to Control-CPT animals. Heart rate was also measured but, no differences between groups were observed (data not shown).

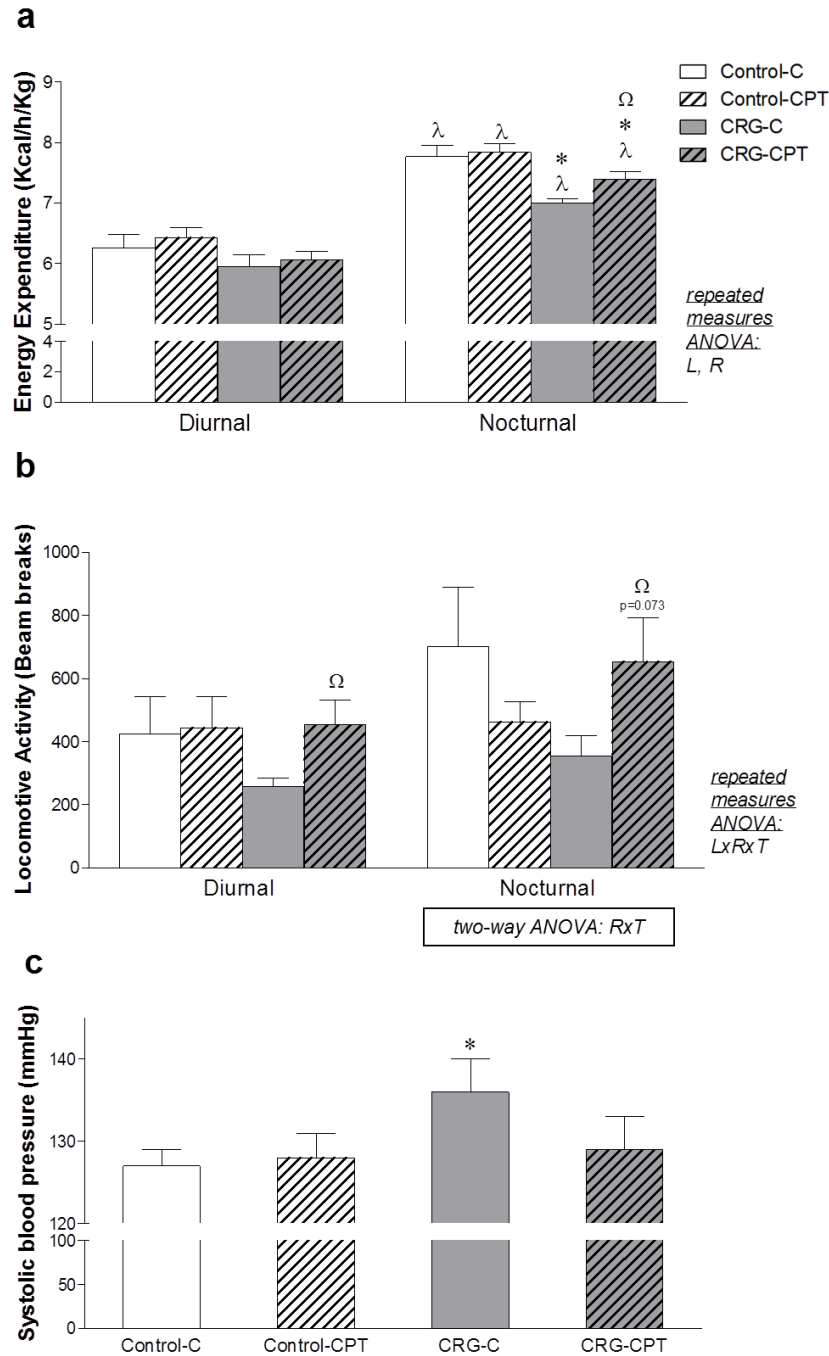


Fig. 6 Energy expenditure (**a**), locomotive activity (**b**) and systolic blood pressure (**c**) of offspring of control dams injected on postnatal day 18 with *Gfp* and *Cpt1am* vectors (Control-C and Control-CPT, respectively) and offspring of calorie restricted dams during gestation injected with *Gfp* and *Cpt1am* vectors (CRG-C and CRG-CPT, respectively), at the age of 3 months (for **a** and **b**) and at the age of 5 months (for **c**). Data are mean \pm S.E.M. (n= 9-11 animals per group). Statistics: *LxRxT*, interaction between light, maternal calorie restriction during gestation and *Cpt1am*-injection; *LxR*, interaction between light and maternal calorie restriction during gestation; *L*, effect of light; *R*, effect of maternal calorie restriction during gestation, ($p < 0.05$, repeated measures ANOVA). λ , nocturnal vs diurnal phase ($p < 0.05$, Paired *t* test). *, CRG vs Control animals; Ω , *Cpt1am* vs *Gfp*-injection, ($p < 0.05$, Student's *t* test). Abbreviations: systolic blood pressure (SBP).

Effects of calorie restriction during gestation and AAV-Cpt1am treatment at an early age on the hepatic expression of leptin receptor and genes related to lipogenesis and inflammation

Fig. 7 shows mRNA expression levels of selected genes in liver of 6-month-old animals. Calorie restriction during gestation resulted in lower hepatic mRNA expression levels of leptin receptor. This effect was partially reverted by AAV-Cpt1am treatment at early stages of life. Therefore, *Obrb* expression levels in CRG-CPT animals were not different compared to their controls (Control-CPT animals). Concerning the expression of lipogenesis related genes, AAV-Cpt1am treatment resulted in lower expression levels of *G6pd* ($p < 0.05$, two-way ANOVA), particularly in CRG animals ($p < 0.05$, Student's *t* test), and of *Acaca*, but only in CRG animals ($p < 0.05$, Student's *t* test, and interactive effect between calorie restriction during gestation and AAV-Cpt1am injection, $p < 0.05$, two-way ANOVA). No significant differences were found concerning *Fasn* expression. AAV-Cpt1am injection resulted in lower expression levels of the inflammation-related genes, *Tnfa* and *Cd68* ($p < 0.05$, two-way ANOVA). In the case of *Tnfa*, the effect was more marked and significant by Student's *t* test in CRG animals ($p < 0.05$). No significant differences between groups were found concerning mRNA expression levels of *Il6* (interleukin 6) and *Il1b* (interleukin 1 beta) (data not shown).

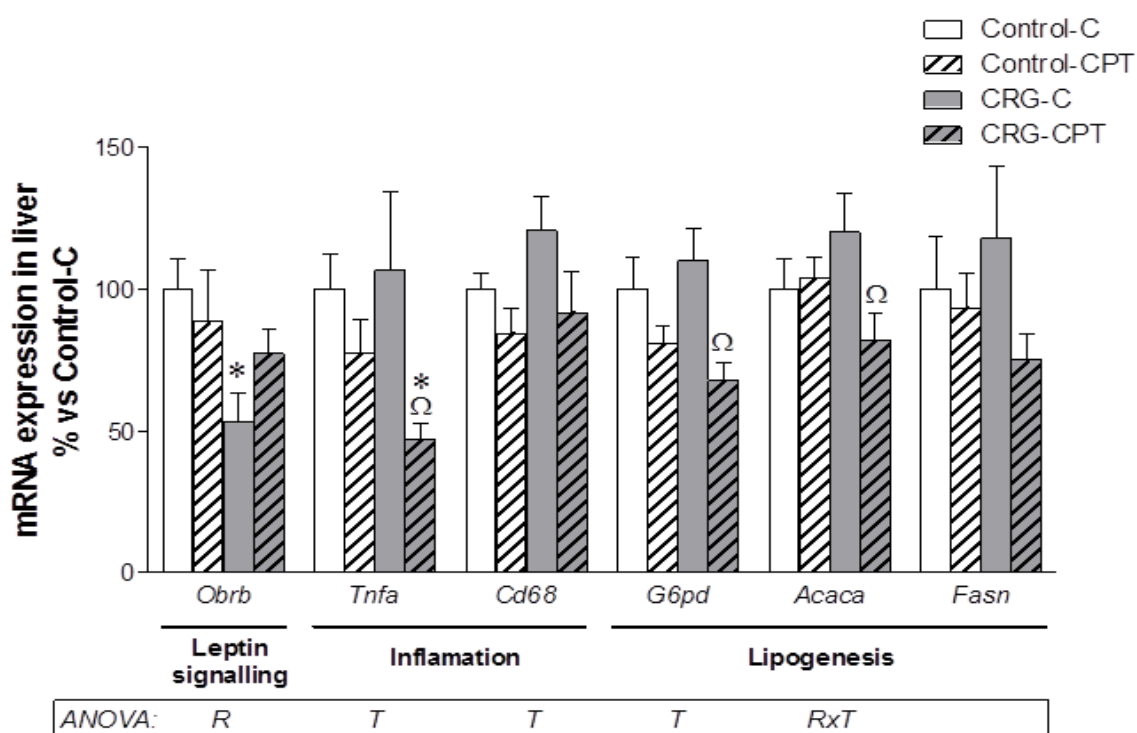


Figure 7. mRNA levels of selected genes in liver from 6-month-old offspring of control dams injected on postnatal day 18 with *Gfp* and *Cpt1am* vectors (Control-C and Control-CPT, respectively) and offspring of calorie restricted dams during gestation injected with *Gfp* and *Cpt1am* vectors (CRG-C and CRG-CPT, respectively). Data are mean \pm S.E.M. ($n = 9-11$ animals per group). Statistics: *RxT*, interaction between maternal calorie restriction during gestation and *Cpt1am*-injection; *T*, effects of *Cpt1am*-injection; *R*, effects of maternal calorie restriction during gestation ($p < 0.05$, two-way ANOVA). *, CRG vs Controls; Ω , *Cpt1am* vs *Gfp*-injection animals, ($p < 0.05$, Student's *t* test). Abbreviations: Acetyl-CoA carboxylase subunit alpha (*Acaca*), cluster of differentiation 68 (*Cd68*), fatty acid synthase (*Fasn*), glucose-6-phosphate dehydrogenase (*G6pd*), long form leptin receptor (*Obrb*) and tumor necrosis factor alpha (*Tnfa*).

Discussion

We have previously described that 20% calorie restriction in rats during the first half of gestation results in metabolic disturbances in the offspring related to the metabolic syndrome (Palou, et al. 2012; Palou, et al. 2010a). These animals, particularly males, show in adulthood altered circulating parameters, impaired insulin and leptin sensitivity and a distorted profile of gene expression in key tissues involved in energy homeostasis (Palou, et al. 2012; Palou, et al. 2010a). Besides, it is accepted that pharmacological or genetic strategies enabling FAO to be enhanced may be beneficial for the treatment of the metabolic disorders related to obesity (Orellana-Gavaldà, et al. 2011; Stefanovic-Racic, et al. 2008). Orellana-Gavaldà *et al.* (Orellana-Gavaldà, et al. 2011) described that AAV-mediated expression of *Cpt1am*, a permanently active mutant form of CPT1A insensitive to malonyl-CoA, protected mice against HF diet-induced obesity, hepatic steatosis and insulin resistance. Interestingly, the use of AAV for gene therapy in humans has become attractive because AAV vectors show wide tissue tropism, efficient transduction, and lack of human pathology (Jazwa A. 2007). Due to the above characteristics and the need for strategies to prevent or treat obesity and its related metabolic disorders, here we aimed to assess whether an AAV-mediated increased expression of *Cpt1am*, and hence enhanced hepatic FAO, at early ages of life is able to revert adverse metabolic malprogramming due to maternal moderate calorie restriction during gestation.

The effectiveness of the AAV-*Cpt1am* vector used here to mediate long-term expression of *Cpt1a* in liver has been previously described (Orellana-Gavaldà, et al. 2011). In our case, injection of AAV was made at early stages of life, postnatal day 18; hence the relative contribution of exogenous *Cpt1a* expression to the total expression was expected to decrease with the animals' growth, particularly with the increase in liver size. In fact, at the age of 6 months, AAV-*Cpt1am* injected animals only showed a small trend to display higher protein levels of CPT1A than animals injected with control-vectors. Higher CPT1A protein levels were only detected in Control-CPT animals compared to Control-C animals in the isolated mitochondrial protein fraction. This is attributable to the fact that AAV can infect both dividing and quiescent cells but persists in an extrachromosomal state without integrating into the genome of the host cell. Therefore, due to the lack of the elements in AAV required for their replication and encapsidation, the number of hepatocytes carrying the *Cpt1am*-vector is expected to become relatively lower compared to the total amount of hepatocytes as animals grew. The presence of green fluorescence in some hepatocytes of adult animals that were injected with AAV-*Gfp*, used as a control, proved the correct insertion of vectors in hepatic cells.

Concerning the lasting effects of maternal undernutrition during gestation, we show here that adult male offspring fed on a HF diet displayed higher fasting levels of insulin, a trend toward higher leptin levels, as well as greater HOMA-IR and L/A ratio compared to controls. In addition to HOMA-IR, L/A ratio has been proposed as a useful, reliable measure of insulin resistance and vascular risk (Finucane, et al. 2009; Satoh, et al. 2004). These results suggest that the offspring of undernourished dams during the first part of gestation presents features of impaired insulin and leptin sensitivity in adulthood, in accordance with our previous results using the same animal model (Palou, et al. 2012; Palou, et al. 2010a). Notably, AAV-mediated *Cpt1am* expression reverted the alterations in plasma insulin levels, HOMA-IR and L/A ratio found in CRG animals. Fasting insulin levels and HOMA-IR were also decreased in control animals injected with AAV-*Cpt1am*. Moreover, CRG-CPT animals also recovered the capacity to respond to feeding/fasting conditions as far as glucose and insulin levels were concerned, which was impaired in CRG animals.

For further study, the potential effects of *Cpt1am*-injection on insulin signalling, hepatic levels of total AKT and its phosphorylated form were determined. It is known that interaction of insulin with its receptor leads to phosphorylation of insulin receptor substrate and initiates a signalling cascade that culminates in the phosphorylation of the serine/threonine-specific protein kinases AKT (Choi and Kim 2010). Once activated, AKT kinases use distinct downstream pathways to modulate metabolism, resulting in enhanced protein synthesis and cell growth, glucose uptake, glycogen synthesis, and lipogenesis (Choi and Kim 2010). Insulin resistance is associated with defective AKT activation (Farese, et al. 2005). However basal levels of pAKT have been found to be increased in HF diet fed mice, and have been related with insulin resistance (Liu, et al. 2009). Here, samples were not obtained under basal conditions, but under feeding conditions at the beginning of the light phase. However, at this time, plasma insulin levels are decreased compared to levels found during the dark phase, when animals consume most of their daily food intake (Sánchez, et al. 2004). Thus, although CRG-C animals showed no changes in hepatic protein levels of total AKT or of the pAKT/total AKT ratio compared to their controls, CRG-CPT rats showed a trend toward increased total protein levels of AKT compared to CRG-C animals, and they displayed a decreased pAKT/total AKT ratio. Hence, the blockade of the continuously elevated AKT-dependent insulin signalling that CRG-CPT animals revealed compared to CRG-C could be tentatively related with an improvement in insulin sensitivity of animals, according to (Liu, et al. 2009); however additional analysis would need to be performed to verify the functioning of this signalling pathway in these animals.

Repeated exposure of the liver to elevated insulin levels has been described to induce increased hepatic TG production (Zammit 2002). In agreement with this, CRG-C animals, which displayed higher fasted insulin levels compared to their controls, also presented a trend to greater amount of lipid vesicles and higher TG content in liver. Notably, CRG-CPT animals, which showed lower fasting insulin levels than CRG-C animals, did not show the mentioned trend, and TG content was similar to that of control animals. Moreover, CRG-CPT animals also showed fewer lipid vesicles than CRG-C animals. The occurrence of higher fasting levels of ketone bodies in CRG compared to their controls may also be related to increased TG accumulation in these animals. In fact, increased levels of plasma ketone bodies have been reported in patients with NAFLD (Sanyal, et al. 2001). Notably, CRG-CPT animals show lower ketone bodies than CRG-C animals. Changes in liver TG content between control and AAV-*Cpt1am* animals may be related to lower expression levels of lipogenesis-related genes, such as *G6pd* and *Acaca*. G6PD catalyses the first, rate-limiting, step of the pentose phosphate pathway. NADPH is the important product of the reaction and is used for the reductive biosynthesis of fatty acids and cholesterol (Singh 2012). NADPH also plays an important function in the protection of the cell against oxidative agents by transferring its reductive power to glutathione disulphide via glutathione disulphide reductase (Singh 2012). Regarding the lipogenic activity of this enzyme, hepatic G6PD has been demonstrated to be regulated by nutritional and hormonal factors, particularly insulin, in function of the demand for NADPH needed for cholesterol and/fatty acid metabolism (Gupte 2010). *G6pd* expression and activity are upregulated in rat and mouse models of obesity, hyperglycemia and hyperinsulinemia, and a role for *G6pd* in the development of insulin resistance in type 2 diabetes has been proposed (Gupte 2010). AAV-*Cpt1am* injected animals, particularly CRG animals, showed lower expression levels of *G6pd* compared to their respective controls. This could be related to lower hepatic lipogenesis activity and the relative protection of these animals to developing insulin resistance. Consistent with these results, CRG-CPT animals also revealed lower mRNA expression levels of *Acaca* compared to their controls. This enzyme is highly expressed in liver and catalyses malonyl-CoA formation, the first step for fatty acid synthesis (Savage, et al.

2006). Moreover, malonyl-CoA also suppresses fatty acid oxidation by inhibiting CPT1A; therefore reduction of *Acaca* may also increase fat oxidation (Savage, et al. 2006).

Differences between groups concerning hepatic glycogen content may also be reflecting differences in insulin sensitivity between groups. Glycogen storage and release of glucose are major functions of the liver, and insulin also plays a main role in the regulation of these processes. Although we did not directly measure glycogen synthesis, the trend to higher hepatic glycogen content in AAV-*Cpt1am* injected animals compared to those injected with the control-vector may suggest increased glycogen synthesis capacity. This could be tentatively related to improved insulin sensitivity in AAV-*Cpt1am* injected animals. Defective hepatic glycogen synthesis would be expected to exacerbate postprandial hyperglycemia, and hence have clinical implications. In this regard, human studies have shown that poorly controlled insulin dependent diabetes mellitus subjects had impaired glycogen synthesis and augmented hepatic gluconeogenesis compared to control subjects (Hwang, et al. 1995).

In addition to insulin, impairment in leptin sensitivity, both central and peripheral, may play a role in the metabolic disturbances associated to undernutrition during gestation (Palou, et al. 2012; Palou, et al. 2010a). Here, even despite the absence of differences concerning body weight between the groups of animals, fasted CRG-C animals showed a trend toward higher leptin levels than controls, suggesting some impairment in leptin sensitivity. In accordance with this, CRG-C animals also displayed lower *ObRb* mRNA levels in liver compared to their controls. Notably, AAV-*Cpt1am* injection at an early age was able to revert, at least partially, the negative lasting effects of maternal calorie restriction on leptin sensitivity, as evidenced by circulating leptin levels and hepatic *ObRb* mRNA levels.

Later undesirable effects associated to severe maternal undernutrition during gestation (both calorie restriction and protein restriction) have also been related to impaired renal function and hypertension (Almeida and Mandarim-de-Lacerda 2005; Vickers, et al. 2000). Hyperinsulinemia may contribute to this alteration but it seems to be particularly caused by alterations in the development of kidneys, leading to a decrease in the number of glomeruli (Almeida and Mandarim-de-Lacerda 2005). In accordance with this, we show here that moderate maternal calorie restriction during gestation also leads to increased blood pressure in adult animals. Interestingly this increase was not seen in AAV-*Cpt1am* injected CRG animals, which showed similar values to those of controls.

The results obtained here regarding the effects of moderate maternal calorie restriction during gestation are generally in agreement with our previous studies using the same conditions (Palou, et al. 2012; Palou, et al. 2010a), as well as with results from other authors using a greater degree of maternal calorie restriction (30%) throughout gestation (Vickers, et al. 2000). However, unlike our previous studies, here we did not observe significant effects of this prenatal moderate restriction on later food intake (data not shown) as animals were able to maintain similar body weight to that of controls, at least during the period studied. Neither did we observe significant differences between control and CRG animals concerning fat accumulation. Nevertheless, CRG-C animals showed a significant increase in the size of adipocytes in rWAT. It is widely known that adipose tissue can respond rapidly and dynamically to alterations in nutrient supply through changes in the number and size of adipocytes, thereby fulfilling its major role in whole body energy homeostasis. It is recognized that adipocyte hypertrophy (an increase in adipocyte volume) prevails in obesity (Sun, et al. 2011), and exposure to a HF diet for a few days has been shown to result in a significant increase in adipocyte size (Sun, et al. 2011). Adipocyte hypertrophy has been previously described in rWAT of adult CRG animals, while hyperplasia is characteristic of the

subcutaneous inguinal depot and associated to the lower sympathetic innervation occurring in these animals (Garcia, et al. 2011). Here, the increased size of adipocytes in CRG animals compared to controls may indicate that these animals have impaired capacity to burn excess energy under HF diet conditions. Interestingly, CRG-CPT animals showed a significant reduction in adipocyte size to levels similar, or even slightly lower, than controls.

Energy expenditure and locomotive activity measures are also in favour of an imbalance of energy homeostasis toward lower energy expenditure in the offspring of calorie restricted dams during gestation. These animals revealed lower energy expenditure during the nocturnal period, which can be associated to a trend to lower locomotive activity. AAV-*Cpt1am* injection in CRG animals reverted the decreased locomotive activity, and this resulted in a partial recovery of energy expenditure levels.

Therefore, increased FAO at early stages of life prevents or ameliorates increased propensity of animals to develop features of the metabolic syndrome associated to maternal undernutrition during gestation. However, increased FAO may be associated with enhanced reactive oxygen species (ROS) production and a consequent inflammatory state (Orellana-Gavaldà, et al. 2011). Thus, to rule out this possibility, inflammation markers were determined.

TNF α is a cytokine involved in systemic inflammation and appears to participate in the induction and maintenance of the subacute inflammatory state associated with obesity. TNF α is overproduced in fatty liver and participates in the development of insulin resistance (Shoelson, et al. 2006). Here we show that CRG-C animals tended toward displaying higher plasma levels of TNF α than their respective controls. These relatively high levels of TNF α could be due to the greater hepatic TG content of these animals and could explain, in part, their insulin resistance. AAV-*Cpt1am* injection in CRG rats led to maintaining TNF α in adulthood at levels similar to their control group. Expression of *Tnfa* in liver was also decreased in AAV-*Cpt1am* injected animals in comparison to animals injected with the AAV-*Gfp*, the decrease being more marked in CRG animals. mRNA expression levels of tissue macrophage marker *Cd68* (Tomita, et al. 1994) were also decreased in AAV-*Cpt1am* injected animals, both control and CRG animals. We did not observe changes in mRNA expression levels of the cytokines *Il6* and *Il1b* in liver (data not shown), which have been described to be overproduced in fatty liver (Shoelson, et al. 2006). This could be due to the fact that here all groups of animals were under HF diet, which could be masking the potential effects of maternal undernutrition on the expression of these genes.

In summary, AAV-*Cpt1am* mediated increased hepatic FAO at a juvenile age is able to revert or prevent some of the metabolic alterations associated to gestational maternal calorie restriction seen in adult animals when exposed to obesogenic conditions, including impaired insulin and leptin sensitivity, and increased systolic blood pressure. This treatment is also able to improve inflammatory markers and prevent the decreased energy expenditure characteristic of these animals. Therefore, enhancement of hepatic FAO at early ages could be considered as a strategy for reverting metabolic disturbances related to developmental malprogramming.

Acknowledgements

We thank Enzo Ceresi for his help in morphological techniques.

Manuscript

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Moderate caloric restriction in lactating rats protects offspring against obesity and insulin resistance in later life

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Endocrinology. 2010;151(3):1030-41

Title Page

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Running title: Caloric restriction in lactating rats and obesity

Statement of financial support: This work was supported by the Spanish Government (grant AGL2006-04887/ALI). Our Laboratory is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, EU Contract: n° FP6-506360). The CIBER de Fisiopatología de la obesidad y nutrición is an initiative of the ISCIII.

Conflict of interest: None

Abstract

We aimed to assess the lasting effects of caloric restriction in lactating rats on body weight and insulin sensitivity of their offspring. Dams were fed with either *ad libitum* standard diet or a 30% caloric restricted diet throughout lactation. After weaning, the offspring were fed with a normal-fat (NF) diet until the age of 15 weeks, and then with a NF or a high-fat (HF) diet until the age of 26 weeks. Blood parameters were measured during *ad libitum* feeding conditions and after 14-h fasting. Food preferences were also measured. In dams, milk leptin concentration and leptin mRNA and protein levels in mammary gland were also determined. Caloric restricted dams showed higher mRNA and protein levels of leptin in mammary gland than controls, without significant changes in milk leptin concentration. The offspring of caloric restricted dams (CRL) ate fewer calories and showed lower body weight gain and fat accumulation under the HF diet than their controls. CRL also maintained unchanged circulating leptin levels under HF diet, while levels increased in controls. In addition, male CRL were resistant to the increase of circulating triglycerides and of the HOMA-IR produced in male controls under HF diet feeding; CRL were also protected against the increased preference for fat-food occurring in females upon HF diet. These results suggest moderate caloric restriction during lactation protects from obesity development in offspring in adult life and from the related metabolic alterations, particularly dyslipidemia, insulin resistance and hyperleptinemia, associated with HF diet feeding.

Keywords: caloric restriction, lactation, obesity, insulin resistance

Introduction

Obesity is a key risk factor in the development of insulin resistance and cardiovascular disease. Both epidemiological and in vivo animal studies agree that the origins of obesity and its related metabolic alterations in adulthood lie not only in genetic heritability and environmental risk factors in adult life, but are also linked to perinatal factors (Taylor and Poston 2007). In this sense, several studies have shown that maternal food restriction during pregnancy may program the risks for adverse health outcomes in the adult offspring (Plagemann 2008; Rasmussen 1998; Wells 2007; Zambrano, et al. 2006; Zubieta and Lonnerdal 2006). This can be explained by the “thrifty phenotype” hypothesis, which postulates that poor fetal nutrition triggers adaptations that improve offspring survival or prepare the fetus to optimize survival in similar nutritional postnatal environments (Fowden, et al. 2005; Gluckman and Hanson 2004; Hales and Barker 2001); however, this may also program subjects to develop obesity and insulin resistance under obesogenic environments in adulthood (Gluckman and Hanson 2004; Wells 2007).

The lasting effects of early postnatal nutrition, particularly during the lactation period have received less attention. In particular, undernutrition during lactation has been studied using several animal models, focusing on caloric and/or protein restriction in the dams (de Moura and Passos 2005; Levin 2006; Rasmussen 1998). In rats, malnutrition produced by protein restriction in the dams during lactation has been associated in the adult offspring with reduced body weight, despite no changes in food intake (de Moura, et al. 2007; de Souza Caldeira Filho and Moura 2000; Fagundes, et al. 2007). Other energy restriction models obtained by severe maternal food restriction during the suckling period (Boxwell, et al. 1995) or by increasing the litter size (Remmers, et al. 2008a; Remmers, et al. 2008b), were also associated with lower body weight and length and also with lower energy intake in adulthood. On the other hand, overnutrition during lactation, induced by reducing litter size, has been associated with higher body weight at weaning and in adulthood and with higher food intake in adulthood (Bassett and Craig 1988; Boullu-Ciocca, et al. 2005; Plagemann, et al. 1999a).

However, most of the studies dealing with the effects of nutrition restriction during lactation have been performed with highly restrictive diets of the dams, such as protein free diets or severe restrictions of food intake (Boxwell, et al. 1995; de Moura, et al. 2007; de Souza Caldeira Filho and Moura 2000; Fagundes, et al. 2007; Moura, et al. 2002; Teixeira, et al. 2002), or by increasing the litter size (Remmers, et al. 2008a; Remmers, et al. 2008b). We hypothesized that 30% caloric restriction during lactation could exert beneficial effects later in life concerning obesity prevention, particularly under obesogenic environments. Thus, here we aimed to assess the effects of moderate caloric restriction (30%) in lactating rats on later body weight, insulin sensitivity, and other energy homeostasis related parameters of their male and female offspring, when exposed to high fat (HF) diet feeding conditions in adulthood. In addition, considering the presence of leptin in maternal milk (Casabiell, et al. 1997; Houseknecht, et al. 1997), its absorption by the stomach of neonate rats (Oliver, et al. 2002; Sanchez, et al. 2005) and the reported protective effects of leptin supply during lactation against obesity and related metabolic alterations in later life (Pico, et al. 2007; Sánchez, et al. 2008), we also aimed to investigate whether changes in the leptin content of milk could be related with the lasting effects of this maternal condition on their offspring.

Materials and Methods

Experimental animals

The study was performed in male and female rats from 12 different litters, following the protocol below. All rats were housed under controlled temperature (22 °C) and a 12 h light–dark cycle (light on from 0800 to 2000), and had unlimited access to tap water and standard chow diet (Panlab, Barcelona, Spain) unless mentioned otherwise. Briefly, twelve virgin female Wistar rats weighing between 200 g and 225 g were mated with male rats (Charles River Laboratories, Barcelona, Spain). After mating, each female was placed in an individual cage. At day 1 after delivery, excess pups in each litter were removed in order to keep 10 pups per dam (five males and five females, when possible) and dams were assigned to either control or caloric restricted group (n=6 in each group). From postnatal day 1 to postnatal day 21, dams were fed either with *ad libitum* standard normal-fat (NF) diet (3 kcal/g; Panlab, Barcelona, Spain) or a 30% caloric restricted diet. Caloric restriction was performed by offering to each dam a daily amount of food corresponding to 70% of the calories that should eat according to its body weight. This amount was calculated considering the calories daily consumed by their control animals under *ad libitum* feeding conditions. Body weight of dams was periodically recorded throughout lactation. Offspring were weaned to solid food at day 21. After weaning, 36 animals from control dams (controls) (18 males and 18 females) and 36 from caloric restricted dams (CRL) (18 males and 18 females) were placed two per cage, paired with another animal of the same group, and fed with standard diet until the age of 15 weeks; then they were distributed into two groups; normal fat (NF) group – animals continued with standard diet (2.9% calories from fat) - and high-fat (HF) group – fed with a chow diet (4.7 kcal/g) with 45% calories from fat (Research Diets, Inc., NJ, USA). HF diet contained 5.5% calories from soybean oil and 39.5% from lard.

In offspring, blood samples were collected under *ad libitum* feeding conditions and after 14-h fasting at the age of 20 weeks (5 weeks after the exposure to half of the animals to HF diet) to analyze circulating parameters. A food preference test was also performed at the age of 12 and 24 weeks to determine dietary preferences. Body weight and food intake was followed until the age of 26 weeks; in addition, body length (from the tip of the nose to the anus) and body fat content (by EchoMRI-700TM, Echo Medical Systems, LLC., TX, USA) were measured without anesthesia (11 weeks after the exposure to half of the animals to HF diet).

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.

Measurements in dams

Sampling

At day 18 after delivery, blood samples (from the saphenous vein, without anesthesia) and milk samples (as previously described (Sanchez, et al. 2005)) were obtained, in both control and caloric restricted dams, under feeding conditions. Plasma was obtained by centrifugation of heparinized blood at 700 g for 10 min. Leptin in plasma and milk was determined using a mouse leptin ELISA kit (R&D Systems, Minneapolis, MN). Total protein levels in milk were measured by the method of Bradford (Bradford 1976).

At weaning, day 21 after delivery, dams were sacrificed by decapitation during the first 2 h after the beginning of the light cycle under feeding conditions. After killing the animals, the mammary gland and the retroperitoneal white adipose tissue (WAT) were rapidly removed, weighed, and frozen in liquid nitrogen and stored at -70°C until RNA and/or protein analysis. Leptin mRNA expression in mammary gland and in WAT, and leptin protein concentration in mammary gland of control and caloric restricted dams were measured.

RNA extraction

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

Real-time quantitative PCR (RT-qPCR) analysis

Real-time polymerase chain reaction (PCR) was used to measure mRNA expression levels of leptin in mammary gland and WAT of control and caloric restricted dams.

0.25 μg of total RNA (in a final volume of 12.5 μl) was denatured at 65°C for 10 min and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20°C for 15 min, 42°C for 30 min, with a final step of 5 min at 95°C a Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain). Each PCR was performed from diluted (1/5) cDNA template, forward and reverse primers (1 μM each), and Power SYBER Green PCR Master Mix (Applied Biosystems, CA, USA). The leptin primers were: forward 5'-TTCACACACGCAGTCGGTAT-3' and reverse 5'-AGGTCTCGCAGGTTCTCCAG-3', with a final product of 186 pb. Real time PCR was performed using the Applied Biosystems StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 min at 95°C , followed by a total of 40 two-temperature cycles (15 s at 95°C and 1 min at 60°C). 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 (C_t) was calculated by the instrument's software (StepOne Software v2.0). Relative gene expression numbers were calculated as a percentage of control rats, using the $2^{-\Delta\Delta C_t}$ method with the beta-actin as reference gene (Pfaffl 2001). The primers used for the beta-actin gene were: forward 5'-TACAGCTTACCACCACAGC-3' and reverse 5'-TCTCCAGGGAGGAAGAGGAT-3', with a final product of 164 pb. All primers were obtained from Sigma Genosys (Sigma Aldrich Química SA, Madrid, Spain).

Western blot analysis

The amount of leptin in mammary gland of control and caloric restricted dams was determined by Western blot. Tissue was homogenized at 4°C in 1:2 (w:v) of phosphate buffered saline (PBS) in a Teflon glass homogenizer. The homogenate was centrifuged at 500 g for 10 min at 4°C and the supernatant was used for total protein and leptin analysis. Total protein content was measured by the method of Bradford (Bradford 1976). For leptin analysis, 350 μg of total protein was solubilized and boiled for 3 min in Laemmli sample buffer containing 5% 2-mercaptoethanol. Then, total protein was fractionated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE; 15% polyacrylamide) and electrotransferred onto a

nitrocellulose membrane (Bio-Rad, Madrid, Spain). Black amide B10 staining provided visual evidence for correct loading and blotting of proteins. After blocking, the membrane was incubated with the primary rabbit polyclonal anti-leptin antibody (Santa Cruz Biotechnology, Inc., CA, USA), diluted 1:1000, and then with the secondary biotinylated anti-rabbit IgG antibody conjugated to a streptavidin-biotinylated horseradish peroxidase complex (Amersham Biosciences, Barcelona, Spain) diluted 1:5000. The immunocomplexes were revealed using an ECLplus (Amersham Biosciences, Barcelona, Spain). Membranes were exposed to Hyperfilm ECL (Amersham Biosciences, Barcelona, Spain). The films were scanned in a Chemigenius BioImaging System (Syngene, UK), and the bands were quantified using the GeneTools Software (Syngene, UK).

Measurements in offspring

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

At the age of 20 weeks (5 weeks after submitting half of the animals to HF diet), blood samples (n = 6-8 animals/group) were collected (in heparinized containers) from the saphenous vein of control and CRL animals, under different feeding conditions: *ad libitum* – animals provided with free access to chow diet – and fasting – animals deprived of food for 14 h. In both conditions, samples were obtained without anesthesia and during the first 2 h of the beginning of the light cycle. Plasma was obtained by centrifugation of heparinized blood at 700 g for 10 min.

Blood glucose concentration was measured by Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Plasma insulin concentration was determined using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (DRG Instruments GmbH, Marburg, Germany) following standard procedures. Plasma leptin concentration was measured using a mouse leptin ELISA kit (R&D Systems, Minneapolis, MN). Commercial enzymatic colorimetric kits were used for the determination of plasma non-esterified fatty acid (NEFA) (Wako Chemicals GmbH, Neuss, Germany) and triglyceride (TG) levels (Triglyceride (INT) 20, Sigma Diagnostics, St Louis, MO, USA).

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

Two-bottle preference test

Food preferences were assessed by a two-bottle preference test as previously described (Sánchez, et al. 2008). Briefly, the rats had to choose between two bottles containing either a carbohydrate (CHO)-rich liquid diet or a fat-rich liquid diet. The two diets had identical caloric density (2.31 kcal/g) and the following ingredients: for the CHO-rich diet, 10 g/100 ml skimmed milk, 40 g/100 ml sucrose, 4 g/100 ml olive oil, and 0.35 g/100 ml xanthan gum (Sigma, Madrid, Spain); and for the fat-rich diet, 10 g/100 ml skimmed milk, 10 g/100 ml sucrose, 17.3 g/100 ml olive oil, and 0.35 g/100 ml xanthan gum. Before the test started, and during a period of 8 d, the rats were habituated to each bottle given individually on alternate days for 1 h, without withdrawing the standard chow diet. The test was started 2 d after the adaptation period. Solid food was withdrawn at the beginning of the light phase. Two bottles

containing pre-weighed quantities of either the CHO- or fat-rich diet were placed side-by-side 4 h after the beginning of the light cycle for 1 h. The bottles were then reweighed, and the intake of each diet was determined and corrected for spillage. Spillage was estimated by weighing small collection plates placed underneath the spout of the bottles. The test was performed at two stages of life, when rats were 12 weeks old (all of them under NF diet) and 24 weeks old (10 weeks after HF diet exposure to half of the animals).

Statistical analysis

Given that the animals studied were from six different litters in each treatment group, the effect of litter was simultaneously factored with all data by repeated measures ANOVA. No interactions between the litter and treatment were found across all the data, thus, data were expressed as mean \pm sem of animals from the six different litters (n = 6-8 animals per group). Multiple comparisons were assessed by repeated measures ANOVA and two-way ANOVA to determine the effects of different factors (age, caloric restriction during lactation, type of diet and feeding conditions). One-way ANOVA followed by Tukey HSD test was used for individual differences between groups to study body weight changes over time. Single comparisons between groups were assessed by Student's t test or Paired t test. $P < 0.05$ was the threshold of significance.

Results

Results in dams

Body weights of control and caloric restricted dams after delivery and at weaning are shown in Table 1. As expected, caloric restricted dams had at the end of the suckling period lower body weight than their controls. These animals also showed a significant reduction in the weight of mammary gland compared with their controls. No statistical differences were found in leptin concentration in plasma and milk and in total protein concentration in milk at day 18 after delivery between both groups of dams (Table 1).

Interestingly, at day 21 after delivery, caloric restricted dams showed significantly higher leptin mRNA expression levels in mammary gland with respect to their controls (Fig. 1), while no statistical differences were found in the mRNA expression of leptin in WAT. Higher leptin mRNA levels in mammary gland of caloric restricted dams were accompanied by higher leptin abundance, both considering specific levels of leptin (the relative abundance of this protein in the mixture) and total leptin levels (referred to the whole mammary gland). These results concerning to the mammary gland correspond to the whole homogenate tissue, hence, the relative contribution of epithelial and adipose cells to leptin production cannot be specified.

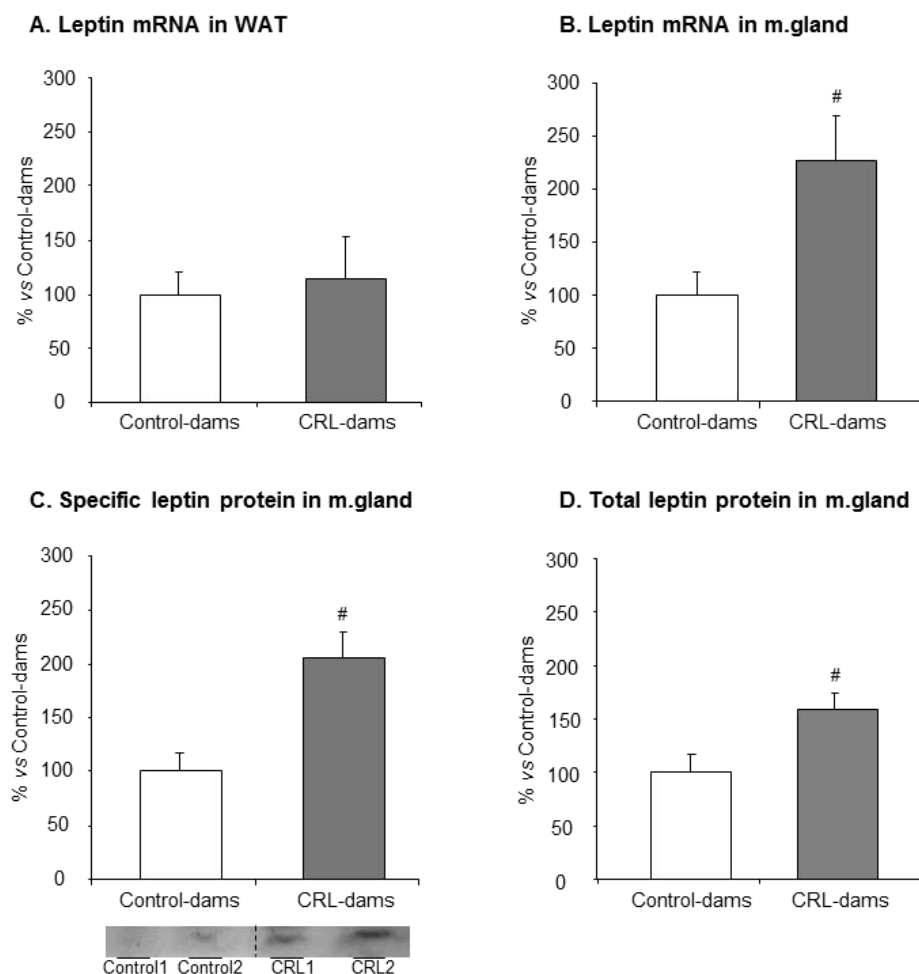


Figure 1. Leptin mRNA expression levels in retroperitoneal WAT (A) and in mammary gland (m.gland) (B), and leptin abundance in mammary gland expressed per mg protein (C) or referred to the whole mammary gland (D) of controls (C-dams) and caloric restricted dams (CRL-dams), under feeding conditions, at day 21 after delivery. A representative western blot of leptin in mammary gland from 2 different dams of each group is also included. Data are expressed as a percentage of the mean value of control dams. Data are mean \pm SEM of 5-6 dams per group. STATISTICS: #, effect of caloric restriction during lactation (Student's t test).

Table 1. Parameters in control and caloric restricted dams.

	Control-dams	CRL-dams
Body weight after delivery (g)	270 \pm 10	260 \pm 12
Body weight at weaning (g)	298 \pm 7 (\uparrow 10%)	244 \pm 11 (\downarrow 6%) #
Weight of mammary gland (g)	17.1 \pm 0.8	10.9 \pm 0.7 #
Plasma leptin concentration (pg/ml)	683 \pm 96	457 \pm 115
Milk leptin concentration (pg/ml)	169 \pm 22	173 \pm 39
Milk protein concentration (mg/ml)	118 \pm 12	93 \pm 4
Milk leptin/total protein ratio	1.49 \pm 0.24	1.92 \pm 0.42

Body weight after delivery (at day 1 of lactation) and at weaning (at day 21 of lactation), weight of the mammary gland at weaning, leptin concentration in plasma and milk, total protein concentration in milk and the milk leptin/total protein ratio (multiplied by 10^9) at day 18 of lactation, in control (C-dams) and caloric restricted dams (CRL-dams) during lactation under feeding conditions. Data are means \pm SEM (n = 6). STATISTICS: # C-dams vs CRL-dams (Student's t test). The percentages in brackets represent the increase or decrease of body weight between day 1 and day 21 of lactation.

Results in the offspring

Body weight and food intake

Moderate caloric restriction in dams during lactation resulted in lower body weight of their offspring, both males and females. This lower body weight was already significant at the age of 6 days and was persistent when animals were growing, with significant differences among the four groups of animals since week 7 of age (one-way ANOVA followed by Tukey HSD test) (Fig. 2). However, differences practically disappeared (no differences by Student's t test) at the age of 26 weeks in those animals maintained under NF diet (Fig. 3A). Nevertheless, CRL animals were more protected against body weight gain when exposed to the HF diet in adulthood (Fig.3A). In fact, after an 11-week period of HF diet feeding, both CRL male and female rats displayed lower body weight than their controls under HF diet (Student's t test).

To ascertain whether lower body weight in CRL animals could be due to lower body length, body length was measured at the end of the follow up, when animals were 26 weeks old. No significant differences were found between body length in CRL animals compared with their controls, either in male or female animals, or under NF or HF diet conditions (data not shown).

Differences in body weight between control and CRL animals can be mainly explained by differences in body fat content (Fig 3B). Body fat content of both control and CRL male animals was higher under HF diet than under NF diet (two-way ANOVA), although the increase by HF diet in CRL rats was not as great as in controls (25% vs 36% respectively). In addition, body fat content was also significantly lower in CRL animals compared with control animals (two-way ANOVA), although differences were only significant under HF-diet (Student's t test). Concerning females, control and CRL rats showed a different response to HF diet feeding (interaction between caloric restriction and diet, two-way ANOVA), since CRL animals were protected against the increase in body fat under HF diet feeding (9% increase in CRL animals versus 31% increase in controls). Notably, CRL female rats had a lower percentage of body fat than their HF diet fed controls, while differences between controls and CRL animals were not observed under NF diet.

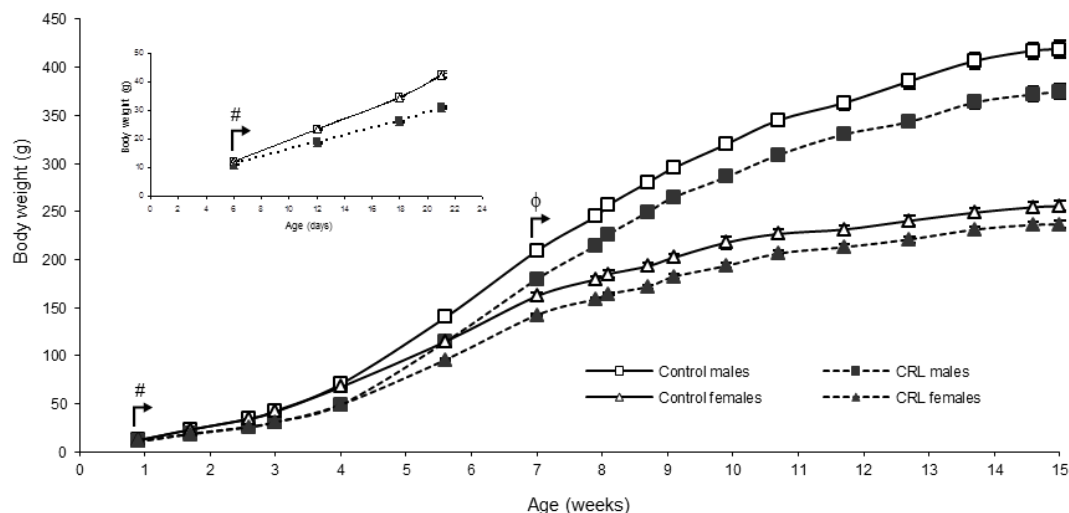


Figure 2. Body weight over time of male and female offspring of control and caloric restricted dams during lactation (CRL) from day 6 until day 105 of age. The inset details body weight during the lactation period and throughout the experiment. Data are expressed as the mean \pm SEM of 18 animals per group. STATISTICS: arrows indicate the age since significant differences were found: #, differences between control and CRL, both in male and female rats; ϕ , differences among the four groups of animals (one-way ANOVA followed by Tukey HSD test)

Cumulative food intake from week 15 to week 26 of age (corresponding to the HF diet feeding period) was also measured to determine whether differences in body weight gain under HF diet between control and CRL animals could be explained by differences in food intake (Fig 3C). As expected, male and female animals exposed to the HF diet ate more calories than when exposed to the NF diet; in males, the increase in total calories was lower in CRL animals than in controls (21% vs 15% respectively), while in females the increase was similar in both groups of animals. Both male and female CRL animals ate fewer calories than their controls, both under NF and HF diet (two-way ANOVA).

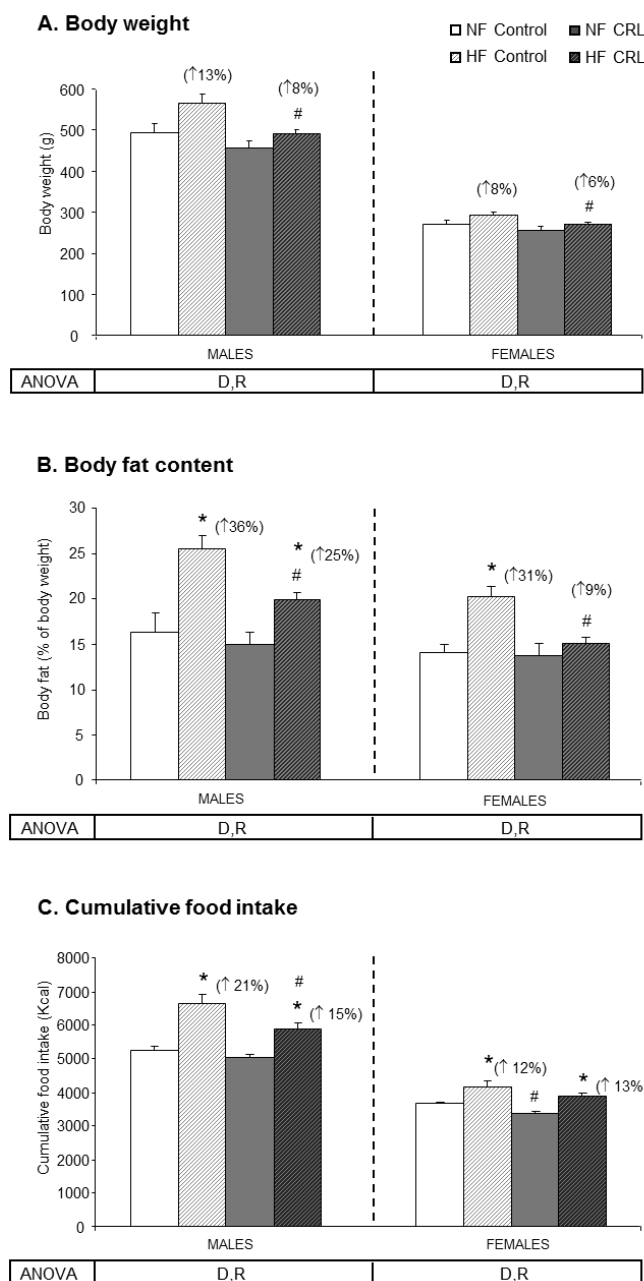


Figure 3. Body weight (A), body fat content (B) and cumulative food intake from day 105 until day 182 (C) of male and female offspring of controls and caloric restricted dams during lactation (CRL) that were fed after day 105 with a NF or a HF diet until the age of 6 months. Results are expressed as the mean \pm SEM of 6 to 12 animals per group. STATISTICS: D, effect of the type of diet; R, effect of caloric restriction during lactation; and RxD, interaction between caloric restriction and diet (two-way ANOVA). *, effect of HF diet (Student's *t* test). #, effect of caloric restriction (Student's *t* test). The percentages in brackets represent the increase as a consequence of the HF diet.

Circulating parameters under fed and fasting conditions

Table 2 shows circulating levels of glucose, insulin, leptin, NEFA and TG of male and female control and CRL animals, under feeding and 14-h fasting conditions at the age of 20 weeks (5 weeks after the exposure of half of the animals to a HF diet).

No significant differences were found in glucose levels as an effect of maternal caloric restriction during lactation. Glucose levels did not significantly decrease in male animals under fasting conditions, with the exception of controls under NF diet (Paired t test). Unlike males, glucose levels in females were significantly lower under fasting conditions compared with fed conditions (ANOVA repeated measures), but the decrease was not significant in controls under NF diet (Paired t test). Insulin levels decreased under fasting conditions in both male and female animals (ANOVA repeated measures). In addition, in male rats, insulin levels were significantly lower in CRL animals than in their respective controls (ANOVA repeated measures), without significant changes in females. No significant differences were found in glucose and insulin levels as an effect of HF diet feeding, either in control or in CRL animals, except control fasted males that showed higher glucose and insulin levels under HF-diet feeding conditions (Student's t test). The HOMA-IR index was calculated to estimate insulin sensitivity (Table 2). This value increased in control male animals under HF diet conditions, while this increase was not found in CRL animals (interaction between caloric restriction and diet, two-way ANOVA). In females, no significant differences were found in the HOMA-IR value as an effect of either HF diet feeding or maternal caloric restriction during lactation.

HF diet feeding resulted in higher circulating leptin levels in both male and female control rats (Student's t test). This increase was also found in CRL male animals under *ad libitum* feeding conditions but not when they were under fasting conditions (Student's t test). However, female CRL animals did not exhibit higher leptin levels when exposed to HF diet, either under feeding or fasting conditions.

Concerning the lipid profile, NEFA levels increased in NF fed control male animals under fasting conditions; however this increase was not found when they were exposed to HF diet. This impairment in the response to fasting conditions was not found in CRL animals exposed to HF diet feeding (Paired t test). Unlike males, both control and CRL female rats increased their NEFA levels under fasting conditions (ANOVA repeated measures), although the highest response (and significant by Student's t test) was found in control animals under NF diet.

Regarding TG, CRL male rats displayed lower plasma TG concentration than their controls (ANOVA repeated measures), and, moreover, they were prevented from the HF-induced increase of TG levels shown in control male animals (Student's t test). Unlike males, no significant differences were found in TG levels among the different female groups (ANOVA repeated measures).

Food preferences

Food preferences were measured at the ages of 12 and 24 weeks with the two-bottle preference test (Fig. 4). When rats were 12 weeks old (Fig. 4A), both control and CRL rats showed a significant preference for CHO-rich food versus fat-rich food (ANOVA repeated measures), which was more evident in males than in females. Moreover, analyzing preferences separately in control and CRL female rats, control rats ate 19% more CHO-rich food than fat-rich (Paired t test) while CRL females only ate 8% more CHO-rich than fat-rich food. When animals were 24 weeks old (Fig. 4B), food preferences were not significantly altered in males under HF-diet

feeding, since both control and CRL animals continued preferring CHO-rich food versus fat-rich food (ANOVA repeated measures). However, female rats under HF diet increased their preference for fat-rich food compared with CHO-rich food (diet interaction with dietary preference, ANOVA repeated measures), but when analyzing control and CRL animals separately, this effect was only significant in control animals (Student's t test). Therefore, significant differences were found between food preferences of control and CRL female rats when were under HF diet feeding (Student's t test).

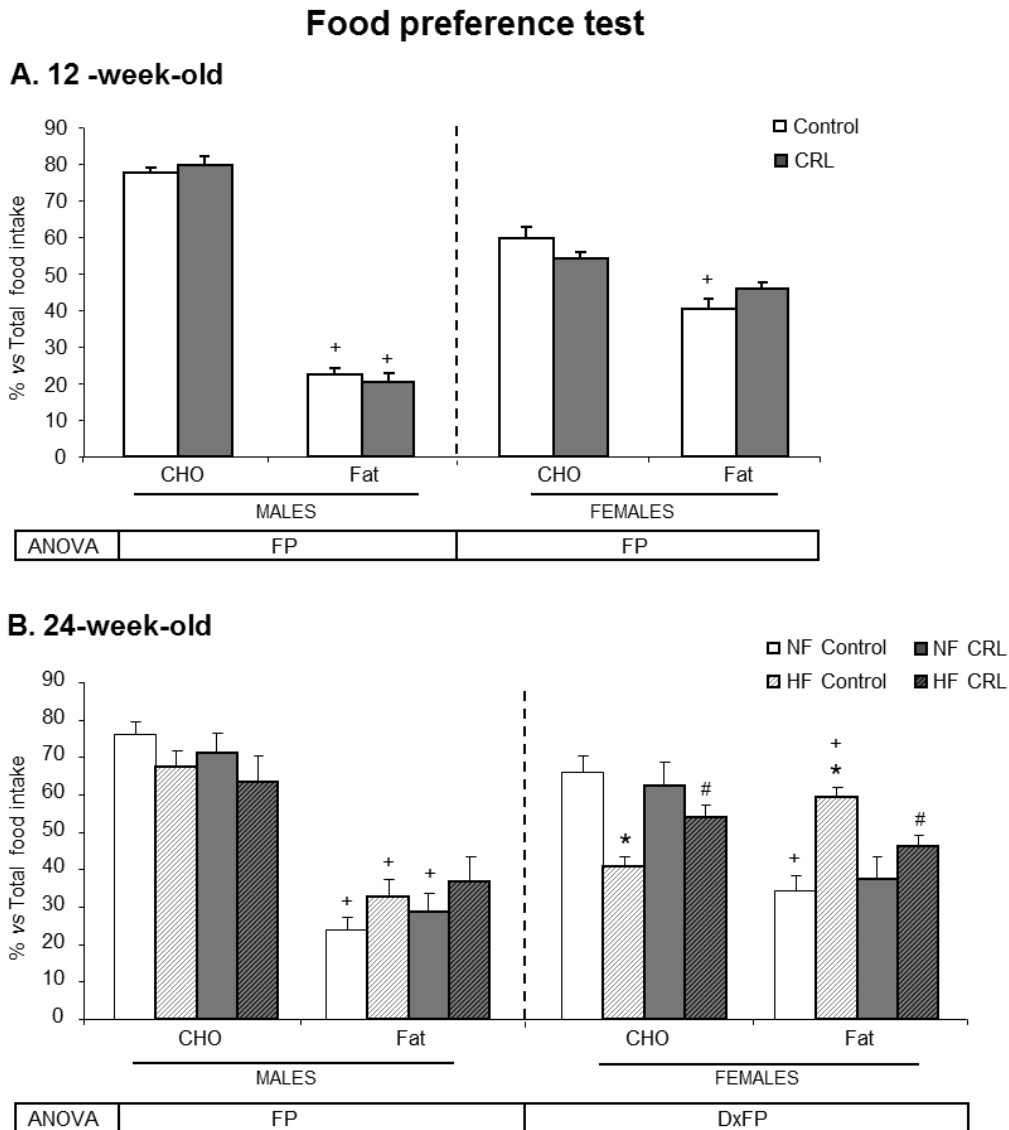


Figure 4. Dietary preferences measured by the two-bottle preference test of the offspring male and female offspring of controls and caloric restricted dams during lactation (CRL) at the age of 12- (**A**) and 24- (**B**) week-old. Bars represent the percentage of the amount of carbohydrate (CHO) rich food and fat rich food with respect to the total food ingested in the choice test. STATISTICS: FP, effect of food preference (two way ANOVA); DxFP, interaction between the type of diet and the food preference (ANOVA repeated measures); +, CHO vs Fat diet (Paired t test); *, effect of HF (Student's t test); #, effect of caloric restriction (Student's t test).

Table 2. Circulating parameters of offspring of controls and caloric restricted dams during lactation

		MALES					FEMALES				
		NF Control	HF Control	NF CRL	HF CRL	ANOVA	NF Control	HF Control	NF CRL	HF CRL	ANOVA
Glucose (mg/dl)	Ad libitum	71.3 ± 1.7	66.6 ± 4.6	73.0 ± 2.0	73.6 ± 5.0		66.0 ± 5.2	75.1 ± 2.4	72.4 ± 5.8	76.4 ± 1.8	F
	14-h fasting	60.3 ± 3.5 ⁺	72.0 ± 2.1 [*]	66.8 ± 4.3	68.0 ± 3.0		65.5 ± 4.1	61.8 ± 2.7 ⁺	62.0 ± 3.6 ⁺	59.3 ± 3.8 ⁺	
Insulin (µg/l)	Ad libitum	4.26 ± 1.52	5.35 ± 0.94	2.78 ± 0.63	2.47 ± 0.43 [#]	F	1.82 ± 0.36	2.01 ± 0.14	1.39 ± 0.48	1.77 ± 0.38	F
	14-h fasting	0.81 ± 0.32 ⁺	1.88 ± 0.30 ⁺ *	0.93 ± 0.25	0.96 ± 0.16 ⁺ #	R	0.37 ± 0.09 ⁺	0.32 ± 0.04 ⁺	0.20 ± 0.01	0.48 ± 0.16 ⁺	
HOMA-IR	14-h fasting	3.09 ± 1.44	7.89 ± 1.17 [*]	3.91 ± 1.22	3.83 ± 0.62 [#]	RxD	1.54 ± 0.49	1.22 ± 0.21	0.73 ± 0.05	1.72 ± 0.53	
Leptin (µg/l)	Ad libitum	5.92 ± 1.38	11.2 ± 1.0 [*]	3.77 ± 0.68	6.22 ± 0.59 ^{*#}	FxR	2.40 ± 0.32	6.53 ± 1.08 [*]	2.63 ± 0.62	3.64 ± 0.58 [#]	RxD
	14-h fasting	2.03 ± 0.59 ⁺	4.98 ± 0.37 ⁺ *	1.59 ± 0.22 ⁺	2.23 ± 0.24 ⁺ #	FxD	0.63 ± 0.07 ⁺	1.60 ± 0.19 ⁺ *	0.63 ± 0.11 ⁺	0.72 ± 0.09 ⁺ #	FxD
NEFAs (µg/l)	Ad libitum	1.14 ± 0.15	1.44 ± 0.11	1.26 ± 0.19	1.46 ± 0.13		0.79 ± 0.18	0.87 ± 0.13	1.34 ± 0.29	1.06 ± 0.19	F
	14-h fasting	1.89 ± 0.16 ⁺	1.58 ± 0.19	2.85 ± 0.60 ⁺	2.16 ± 0.28 ⁺	FxD	1.34 ± 0.12 ⁺	1.16 ± 0.13	1.45 ± 0.23	1.16 ± 0.15	
Triglycerides (mg/ml)	Ad libitum	1.24 ± 0.11	2.19 ± 0.27 [*]	1.17 ± 0.27	1.05 ± 0.12 [#]	F	0.53 ± 0.10	0.56 ± 0.08	0.56 ± 0.09	0.53 ± 0.04	
	14-h fasting	1.53 ± 0.43	1.10 ± 0.27 ⁺	0.49 ± 0.14 ⁺	0.62 ± 0.13 ⁺	R	0.50 ± 0.04	0.41 ± 0.04	0.54 ± 0.08	0.45 ± 0.04	

Blood glucose and plasma insulin, leptin, non-esterified fatty acid (NEFA) and triglycerides concentration under ad libitum feeding or 14 h fasting conditions, and HOMA-IR index at the age of 20 weeks of male and female offspring of controls and caloric restricted dams during lactation (CR), that where fed with a NF or a HF diet. Results are expressed as the mean ± SEM of 6 to 8 animals per group. STATISTICS: R, effect of caloric restriction during lactation; F, effect of feeding conditions; D, effect of the type of diet; and RxD, interaction between caloric restriction and diet; FxR, interaction between feeding conditions and caloric restriction; and RxD, interaction between caloric restriction and diet (ANOVA repeated measures). ⁺, *ad libitum* vs. Fasting (Paired t test). ^{*}, NF vs. HF diet; [#], Control vs. CR (Student's t test).

Discussion

Epidemiological and animal model studies have shown a link between the perinatal nutritional environment and the susceptibility to suffer obesity and metabolic alterations in adult life (Cripps, et al. 2005; Gluckman and Hanson 2004; Plagemann 2008). In particular, conditions such as undernourishment during gestation and overfeeding during early postnatal life have been described to have a strong negative impact in adulthood (Martorell, et al. 2001). The present study was conducted to determine the effects of moderate maternal caloric restriction (30%) during lactation on offspring body weight, insulin sensitivity, and other physiological parameters related with energy homeostasis, including energy intake and food preferences, after HF-diet feeding in adulthood and to find out whether there is a sex-associated response.

Our results show that moderate caloric restriction (30%) during lactation resulted in lower body weight of male and female offspring which was already manifest as of the first days of life; this could be attributed to lower maternal milk supply as previously described in other models of food restriction during lactation (Bautista, et al. 2008; Grigor, et al. 1987). This lower body weight was maintained while the animals were growing, but was gradually attenuated with aging. The effect of maternal caloric restriction was more evident when rats were exposed to the HF diet, although in a sex-dependent manner. CRL male and female animals under HF diet gained less body weight and accumulated lower body fat than their controls, but interestingly, CRL females were almost resistant to body weight and body fat accumulation under HF diet. The effect in male animals can be explained, at least in part, by lower food intake. In fact, the increased intake of calories under HF diet was lower in CRL animals versus their controls. However, in CRL female rats under HF diet exposure, the difference cannot be explained by lower induction of food intake. In fact, energy intake in CRL female animals was lower than that of control animals, but the increase under HF diet was not different to that of control animals under this diet.

Despite changes in body weight, maternal caloric restriction did not affect body length of the animals. Thus, the results of this study suggest that moderate maternal caloric restriction during lactation protects against diet-induced obesity in rats but does not impede catching up the normal body length and weight under standard, NF diet conditions. These results contrast with those obtained by increasing the litter size (Remmers, et al. 2008a; Remmers, et al. 2008b), since these animals maintained lower body weight and length than their controls. Other models of nutrient restriction during lactation obtained by a 50% or a 65% maternal protein restriction (Fagundes, et al. 2007; Zambrano, et al. 2006) also showed more severe results with also lower body weight and fat content of restricted offspring. This finding underscores the importance of the type and degree of restriction on the resulting effects.

Leptin, a hormone primarily produced by the adipose tissue, and the pancreatic hormone insulin have elicited great attention as important modulators of feeding patterns and energy expenditure at the hypothalamic level (Schwartz, et al. 2000). Both proteins are closely related with the regulation of energy balance at different levels (Cripps, et al. 2005; Moura, et al. 2002), and their circulating levels, together with their adaptations under fed/fasting conditions, may be considered as markers of metabolic health and of appropriate body weight and food intake control. Programming of the leptin and insulin systems by early life nutrition may contribute to the long-term maintenance of energy homeostasis. Sex-dependent differences have been described concerning the response of these systems to dietary stressors (Priego, et al. 2009b; Priego, et al. 2008; Woods, et al. 2003). Regarding insulin, diet-induced obesity has

been associated with an impairment of insulin sensitivity and the development of Type II diabetes (Woods, et al. 2004). Interestingly, male rats have been found to exhibit a higher tendency for hyperinsulinemia induced by hyperlipidic diets than females (Priego, et al. 2008). In this sense, we also found an increase in the HOMA-IR value occurring in male control rats under HF diet but not in female rats. Nevertheless, the male offspring of caloric restricted dams during lactation appeared to be protected against HF diet-induced insulin resistance. No differences were found in the HOMA-IR value between control and CRL female animals, although females did not show increased HOMA-IR index under HF diet but maintained similar values to those of NF diet fed animals. Thus, these results concerning HOMA-IR suggest that moderate maternal energy restriction throughout lactation may confer protection against diet-induced insulin resistance in male offspring in later life, which are more prone to suffer this metabolic alteration under HF diet conditions, as previously described (Priego, et al. 2008). Maternal isocaloric protein restriction during lactation has also been shown to result in higher insulin sensitivity in their offspring: in concrete, 21-day-old pups from 60% protein restricted dams during lactation displayed lower fasted insulin levels and an up-regulation of the insulin receptor beta (Martin-Gronert, et al. 2008); moreover, offspring from 50% protein restricted dams showed lower area under the curve for glucose and insulin in a glucose tolerant test in adulthood (Zambrano, et al. 2006).

Concerning the leptin system, moderate maternal caloric restriction during lactation did not seem to have lasting effects on circulating leptin levels in the offspring; when animals were under NF diet, a tendency to lower levels was only found in male animals. The effect of caloric restriction was more evident under dietary stressors, such as HF diet exposure, particularly in female rats. These animals maintained the leptin levels of NF fed animals when exposed to HF diet, in accordance with the maintenance of the amount of fat. This is of interest because the presence of low leptin levels has been associated with an improvement of leptin sensitivity (Sánchez, et al. 2008), whereas an increase of circulating leptin levels may contribute to the dysregulation of energy balance that occurs associated to HF diet feeding and with age and involves impairment of the fasting-induced suppression of leptin production (Iossa, et al. 1999; Pico, et al. 2007; Sánchez, et al. 2008). Thus, moderate caloric restriction to lactating dams may help maintain lower leptin levels in the offspring on later life under HF diet feeding conditions, and could help these animals be more responsive to adaptive changes in leptin levels. Nevertheless, plasma leptin concentration decreased in both control and CRL animals under fasting conditions, although, under these conditions, CRL animals also maintained lower plasma leptin levels than their controls. More restrictive maternal caloric restriction during lactation (67%) also resulted in lower circulating leptin levels in offspring at the age of 21 days (Boxwell, et al. 1995); in addition, another energy restriction model during lactation obtained by increasing litter size also resulted in lower circulating leptin levels in their offspring, already since the age of 10 days (Remmers, et al. 2008a). On the other hand, a 65% maternal isocaloric protein restriction during lactation resulted in lower leptin levels of their offspring at the age of 12 days compared with controls, but levels became higher at the age of 21 days (Teixeira, et al. 2002).

Together with circulating insulin and leptin levels, blood lipid profile can be considered as indicators of metabolic health. Male rats have been described to be more prone to suffer a higher increase in circulating lipids under HF diet, and this has been linked to the detrimental effects of the overweight in this gender in comparison with females (Priego, et al. 2008; Regitz-Zagrosek, et al. 2006). Our results also showed an impairment of the NEFA response to fasting and also higher TG levels under feeding conditions in control male rats under HF diet feeding, while these effects were not found in CRL male rats under HF diet. Thus, plasma lipid profile of CRL male rats, with higher NEFA response to fasting under HF diet and with lower

circulating TG levels, may contribute to explain their increased insulin sensitivity.

Programming food preferences, as a part of the feeding behavior, may contribute to body weight control and to the development of obesity. This is particularly relevant in humans where obesity development is generally associated with increased appetite and preference for highly caloric food, together with a sedentary life, among other factors (Rissanen, et al. 2002). Here, we show that caloric restriction to lactating dams affected food preferences of adult female offspring when exposed to a HF diet. Female animals, compared with males had higher preference for fat-rich food, as previously described (Bellinger, et al. 2004; Eckel and Moore 2004; Roca, et al. 1999). Moreover, these animals, when exposed to HF diet, increased their preference for fat-rich food compared with CHO-rich food. However, this preference for fat-rich food was not observed in CRL female animals; these animals even had a slightly lower preference for fat-rich food than for CHO-rich food. Less preference for fat-rich food could entail better feeding behavior, considering that these animals are exposed to a HF diet. Even though CRL female animals showed a lower preference for fat-rich food when were able to chose between CHO and fat food, this did not prevent that these animals experienced a similar increase in the intake of calories to that of control animals when exposed to HF diet. Other studies related with modifications of perinatal nutrition have described changes in food preferences in later life. For example, oral supplementation with leptin during lactation protects animals against diet induced obesity and enhances preferences for CHO-rich foods than for fat-rich foods (Sánchez, et al. 2008). On the other hand, maternal protein restriction during fetal stage is associated with an obesogenic phenotype in adulthood and causes an increase of fat food preferences (Bellinger, et al. 2004). Thus, nutritional changes during critical stages of development, such as fetal life or lactation, may program the control mechanisms of appetite and food preferences, which are important in the susceptibility to suffering obesity later on; therefore, the study of this perinatal programming may provide effective tools to curb obesity and related metabolic diseases.

Mechanisms involved in the developmental programming by maternal caloric restriction during lactation, as well as specific changes occurring in milk composition under these conditions are currently unknown. Leptin is a hormone naturally present in breast milk (Casabiell, et al. 1997; Houseknecht, et al. 1997) and previous studies have shown that supplementation of neonate rats with physiological doses of leptin during lactation protects against the development of overweight in adulthood (Pico, et al. 2007) and improves insulin and leptin sensitivity (Sánchez, et al. 2008); in addition, a negative correlation has been found in humans between maternal milk leptin levels and body weight gain of infants (Miralles, et al. 2006). Thus, considering the importance of leptin supply during lactation, we postulated that changes in leptin concentration in milk as an effect of caloric restriction could account, at least in part, for these metabolic programming effects. Leptin levels in maternal milk have been shown to be positively correlated with maternal adiposity and body mass index (Miralles, et al. 2006), however, little is known about the regulation of milk leptin concentration by maternal nutritional factors. Of interest, some authors have described an increase in leptin content in milk as a consequence of maternal protein restriction in lactating dams (Bautista, et al. 2008). It must be pointed out that leptin in milk comes from both the maternal blood source and its production in the mammary gland (Casabiell, et al. 1997). Blood leptin seems to be differentially regulated during lactation. Ferreira et al. (Ferreira, et al. 2007) have shown that isocaloric protein restricted dams during the first 14 days of lactation display higher serum leptin levels than their controls, despite having lower body weight. Our results, showed no statistical differences in plasma leptin levels between control and caloric restricted dams, suggesting maintenance of the adequate circulating leptin levels in these rats. On the other hand, concerning leptin production by the mammary gland, we found that caloric restricted

dams had, at weaning, higher mRNA and protein levels of leptin in mammary gland compared with their controls. Moreover, although caloric restricted dams exhibited a reduction in the weight of mammary gland compared with controls, total leptin abundance estimated in the whole mammary gland was still higher than in their controls. The increased expression of leptin was tissue-specific, since no changes were observed in the retroperitoneal WAT of these dams. However, milk leptin concentration in caloric restricted dams measured before weaning (at day 18 after delivery) was similar to that of control animals. At present, the postulated contribution of milk leptin to the lasting effects of moderate caloric restriction during lactation in the offspring must be considered as speculative, but this is an interesting subject to be considered for future investigations.

All in all, these results showing the potential benefits of moderate maternal caloric restriction during lactation fit with the relatively recent idea that promoting catch-up growth in low birth weight infants may not be beneficial for their long-term outcome (Fagerberg, et al. 2004). However, it must be stressed that we should be cautious in extrapolating these results to humans due to differences between species, particularly in the different timing of birth relative to development.

In conclusion, here we show that moderate maternal caloric restriction during the lactation period has lasting gender-specific effects in the offspring in adult life. Regardless of these gender-specific particularities, the offspring from caloric restricted dams become less prone to diet-induced obesity and related metabolic alterations in adult life, particularly dyslipidemia, insulin resistance and hyperleptinemia.

Manuscript

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Moderate caloric restriction in
lactating rats programs their offspring
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J Nutr Biochem. 2011;22(6):574-84

Title page

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Statement of financial support: The research leading to these results was supported by the Spanish Government (grant AGL2009-11277). The authors' Laboratory is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, EU Contract: nu FP6-506360). The CIBER de Fisiopatología de la obesidad y nutrición is an initiative of the ISCIII.

Conflict of interest: None

Abstract

We aimed to assess the lasting effects of moderate caloric restriction in lactating rats on the expression of key genes involved in energy balance of their adult offspring, and their adaptations under high-fat (HF) diet. Dams were fed with either *ad libitum* standard diet or a 30% caloric restricted diet throughout lactation. After weaning, the offspring were fed with a normal-fat (NF) diet until the age of 15 weeks, and then with a NF- or a HF-diet until the age of 28 weeks, when they were sacrificed. Body weight and food intake were followed. Blood parameters and the expression of selected genes involved in food intake and fat accumulation in hypothalamus and white adipose tissue (WAT), respectively, were analysed. The offspring of caloric restricted dams (CRL) ate fewer calories and showed lower body weight gain under HF-diet than their controls. CRL males were also resistant to the increase of insulin and leptin occurring in their controls under HF-diet conditions, and HF-diet exposed CRL females showed lower circulating fasting TG levels than their controls. In the hypothalamus, CRL males had higher *Obrb* mRNA levels than controls, and CRL females displayed greater *Insr* mRNA levels than controls and decreased *Npy* mRNA levels when exposed to HF diet (contrary to controls). CRL males maintained WAT capacity of fat uptake and storage and of fatty acid oxidation under HF-diet, whereas these capacities were impaired in controls; female CRL showed higher WAT *Obrb* mRNA levels than controls. These results suggest that 30% caloric restriction in lactating dams ameliorates diet-induced obesity in their offspring by enhancing their sensitivity to insulin and leptin signaling, but in a gender-dependent manner.

Key words: caloric restriction, lactation, obesity, insulin and leptin sensitivity, hypothalamus, adipose tissue

Introduction

Epidemiological and experimental studies have described that the programming of energy balance already begins in very early development. Indeed, particular conditions in the nutritional environment during the perinatal period may lead to adjustments in the physiology of humans and animals, with lasting effects in adulthood (Bertram and Hanson 2001; Cripps, et al. 2005; Gluckman and Hanson 2004; McMillen, et al. 2005).

The hypothalamus plays a major role in the regulation of energy balance, producing many orexigenic and anorexigenic peptides that stimulate or inhibit food intake in response to different factors, such as circulating hormones like leptin and insulin (Wilding 2002). The brain is particularly sensitive to external factors during the early period of life (McMillen, et al. 2005); in this sense, many studies have shown the importance of nutritional factors during this period in the programming of appetite behavior in the adult life (García, et al. 2010; Pico, et al. 2007; Sánchez, et al. 2008). Perinatal nutrition leading to changes in the control of body weight and food intake has been associated with developmental programming of structural and functional changes in hypothalamus affecting the expression of key genes involved in food intake and energy balance (García, et al. 2010; Pico, et al. 2007)

On the other hand, the adipose tissue is recognized to have different functions that are important in the regulation of energy balance and substrate metabolism (Palou, et al. 2008). Both fat storage and fat mobilization processes normally occur in the white adipose tissue (WAT) under the habitual food intake/fasting patterns of feeding, and allow the maintenance of energy homeostasis (Goodman, et al. 1980; Palou, et al. 1981; Palou, et al. 2009). Alterations of these processes by different stressor factors such as high fat (HF) diet feeding or food deprivation promote dysregulation in the overall control of fat deposition affecting energy balance (Palou, et al. 2008; Priego, et al. 2008). It is clear that the maintenance of nutrient homeostasis under different feeding conditions and the metabolic response to these situations involve hormonal and metabolic adaptations which are accompanied by changes in gene expression (Palou, et al. 2009; Palou, et al. 2009c; Priego, et al. 2008).

In mammals, maternal nourishment establishes the first nutritional environment of their offspring; thus, changes in maternal nutrition may program alterations in the metabolism of offspring later on in life (Armitage, et al. 2005; Dewey 1998a; Levin 2006). In this sense, strong evidence has linked low birth weight with the susceptibility to suffer obesity in adult life. This has allowed to establish the thrifty phenotype hypothesis (Cripps, et al. 2005; Gluckman and Hanson 2004; Wells 2007), which relates malnutrition during the fetal stage and the adaptations for survival experienced by the offspring. On the other hand, early postnatal nutrition may also cause differential programming of energy homeostasis. In particular, malnutrition produced by protein restriction in lactating dams has been associated with reduced body weight in the adult offspring, despite no changes in food intake (de Moura, et al. 2007; de Souza Caldeira Filho and Moura 2000; Fagundes, et al. 2007). Other models of energy restriction obtained by severe food restriction in lactating dams (Boxwell, et al. 1995) or by increasing the litter size (Remmers, et al. 2008a; Remmers, et al. 2008b) have also been associated with lower body weight and lower food intake in their adult offspring. In this sense, we have also recently described in rats that moderate caloric restriction (30%) in lactating dams protects their offspring against obesity as well as against insulin resistance in adulthood (Palou, et al. 2010b). Most of these studies analyzing the lasting effects of maternal caloric restriction in offspring have mainly focused on the changes in body weight, food intake and/or circulating hormone levels (Moura, et al. 2002; Remmers, et al. 2008a; Šefčíková 2002; Teixeira, et al.

2002). However little is known about the changes occurring at gene expression level in key tissues involved in energy balance as a consequence of maternal food restriction during suckling period that may be determinant of the lower propensity to obesity. We hypothesized that early postnatal food restriction may induce developmental programming of hypothalamic and WAT gene expression of key factors involved in the regulation of energy balance. Thus, we aimed to gain further insight into the mechanisms that could underlie the substantial outcome in the male and female offspring of moderate caloric restricted (30%) lactating dams by analyzing the mRNA expression levels of selected genes involved in the regulation of food intake and fat accumulation in the hypothalamus and WAT, respectively, and establishing their relationship with energy homeostasis-related parameters when these animals were exposed in adulthood to HF diet conditions. In addition, considering that the adaptation to fed/fasting conditions may be impaired in obese animals, HF diet exposed animals were studied under both feeding and fasting conditions.

Materials and methods

Experimental animals

The study was performed in male and female rats from 12 different litters, following the protocol below. All rats were housed under controlled temperature (22°C) and a 12 h light–dark cycle (light on from 08:00 to 20:00), and had unlimited access to tap water and standard chow diet (Panlab, Barcelona, Spain) unless mentioned otherwise. Briefly, twelve virgin female Wistar rats weighing between 200 g and 225 g were mated with male rats (Charles River Laboratories, Barcelona, Spain). After matching, each female was placed in an individual cage. At day 1 after delivery, excess pups in each litter were removed in order to keep 10 pups per dam (five males and five females, when possible) and dams were assigned to either control or caloric restricted group (n=6 in each group). The control group was fed *ad libitum* with standard diet, while the caloric restricted group was provided daily with a 30% caloric restricted diet throughout lactation, starting on day 1 after delivery and ending at weaning (day 21) as previously described (Palou, et al. 2010b). After weaning, 36 animals from control dams (controls) (18 males and 18 females) and 36 from caloric restricted dams (CRL) (18 males and 18 females) were placed two per cage, paired with another animal of the same group, and fed with standard diet until the age of 15 weeks; then they were distributed into two groups; normal fat (NF) group – animals (n=6/group) continued with standard diet (2.9% calories from fat) - and high-fat (HF) group – animals (n=12/group) fed with a chow diet (4.7 kcal/g) with 45% calories from fat (Research Diets, Inc., NJ, USA). HF diet contained 5.5% calories from soybean oil and 39.5% from lard. Body weight and food intake were followed.

At the age of 28 weeks, NF diet and a half of the HF diet fed rats (n=6/group) were killed under *ad libitum* feeding conditions, while the second half of HF diet group (n=6/group) was killed after 12-h fasting. All animals were sacrificed by decapitation during the first 2 h of the beginning of the light cycle and on different consecutive days (including animals from each group every day). Blood samples were collected in heparinized containers, then centrifuged at 700 g for 10 min to obtain the plasma, and stored at -20°C until analysis. The hypothalamus and the main WAT depots (retroperitoneal, mesenteric, gonadal and inguinal) were rapidly removed, weighed, frozen in liquid nitrogen and stored at -70°C until ulterior studies. The hypothalamus was harvested by using the following landmarks, i.e., frontal edge of the optical chiasm, lateral sulci, caudal edge of the mammary bodies, and a depth of 2 mm. Although

different WAT depots were sampled to be weighed, the retroperitoneal depot was selected as representative to be analyzed for gene expression.

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

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

Blood glucose concentration was measured by Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Plasma insulin concentration was determined using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia AB, Uppsala, Sweden) following standard procedures. Plasma leptin concentration was measured using a mouse leptin ELISA kit (R&D Systems, Minneapolis, MN). Circulating triglycerides were measured by commercial enzymatic colorimetric kit (Triglyceride (INT), Sigma Diagnostics, St Louis, MO, USA).

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

RNA extraction

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

Real-time quantitative PCR (RT-qPCR) analysis

Real-time polymerase chain reaction (PCR) was used to measure mRNA expression levels of neuropeptide Y (*Npy*), proopiomelanocortin (*Pomc*), long form leptin receptor (*Obrb*), insulin receptor (*Insr*), and suppressor of cytokine signaling 3 (*Socs3*) in hypothalamus, peroxisome proliferator activated receptor gamma 2 (*Ppar γ 2*), acetyl-coenzyme A carboxylase alpha (*Acc1*), glycerol-3-phosphate acyltransferase (*Gpat*), glucose transporter 4 (*Glut4*), lipoprotein lipase (*Lpl*), the free fatty acid transporter *Cd36*, muscle carnitine palmitoyltransferase 1a (*Cpt1m*), *Obrb* and *Insr* in rWAT. 0.25 μ g of total RNA (in a final volume of 5 μ l) was denatured at 65°C for 10 min and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20°C for 15 min, 42°C for 30 min, with a final step of 5 min at 95°C in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain). Each PCR was performed from diluted cDNA template, forward and reverse primers (1 μ M each), and Power SYBER Green PCR Master Mix (Applied Biosystems, CA, USA). Primers were obtained from Sigma (Madrid, Spain) and sequences are described (García, et al. 2010; Palou, et al. 2009; Priego, et al. 2008), except for the *Socs3* sequences that were: forward 5'-ACTGAGCCGACCTCTCTCCT-3' and reverse 5'-CCCCTCTGACCCTTTCTTTG-3'. Real time PCR was performed using the Applied Biosystems StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) with the

following profile: 10 min at 95°C, followed by a total of 40 two- temperature cycles (15 s at 95°C and 1 min at 60°C). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.0) and the relative expression of each mRNA was calculated as a percentage of NF control rats under *ad libitum* feeding conditions, using the $2^{-\Delta\Delta C_t}$ method with the beta-actin and 18S as reference genes (Pfaffl 2001). All primers were obtained from Sigma Genosys (Sigma Aldrich Química SA, Madrid, Spain).

Statistical analysis

Given that the animals studied were from six different litters in each treatment group, the effect of litter was simultaneously factored with all data by repeated measures ANOVA. No interactions between the litter and treatment were found across all the data, thus, data were expressed as mean \pm sem of animals from the six different litters. Multiple comparisons were assessed by repeated measures ANOVA and two-way ANOVA. Single comparisons between groups were assessed by Student's t test or Paired t test. $P < 0.05$ was the threshold of significance.

Results

Weight-related parameters and food intake

As shown in Figure 1A, moderate caloric restriction in dams during lactation resulted in lower body weight of their offspring in adulthood, both males and females (two-way ANOVA). This effect was more pronounced under HF diet feeding; in fact, by individual comparison, there were no statistical differences between control and CRL rats under NF diet (Student's t test). This lower body weight can be explained, at least in part, by lower food intake. Both male and female CRL animals ate fewer calories than their controls, both under NF and HF diet (Figure 1B).

Differences in body weight between control and CRL animals can be attributed to the size of fat depots (Table 1). The inguinal and mesenteric adipose tissue weights in both CRL male and female animals and the weight of the gonadal depot in CRL females were lower than in control animals (two-way ANOVA). Differences were generally found both under NF and HF diet conditions, but were more marked under HF diet. Moreover, CRL males presented lower adiposity index than their controls both under NF and HF diet (two-way ANOVA), and this effect was also more pronounced under HF (Student's t test).

Circulating parameters under fed and fasting conditions

Table 2 shows circulating glucose, insulin, leptin, and TG levels of male and female control and CRL animals exposed to NF and HF diet, under feeding conditions, as well as after 12-h fasting conditions in HF-diet exposed animals. No significant differences were found in glucose levels between control and CRL rats as an effect of the caloric restriction or HF diet feeding (two-way ANOVA). Fasted rats presented lower glucose levels than fed animals (two-way ANOVA). Control male animals, but not CRL males, showed increased insulin

concentration under feeding conditions when exposed to HF diet (two-way ANOVA); in fact, under HF diet conditions, CRL males had lower insulin levels than their controls both under feeding and fasting conditions (two-way ANOVA). In females, no changes were found as an effect of caloric restriction or HF diet feeding (two-way ANOVA). Both males and females showed a decrease in insulin levels after 12-h fasting (Student's *t* test). Interestingly, under HF diet, control males showed a tendency to higher insulin resistance index (HOMA-IR) versus CRL rats ($p=0.062$, Student's *t* test) (Figure 2).

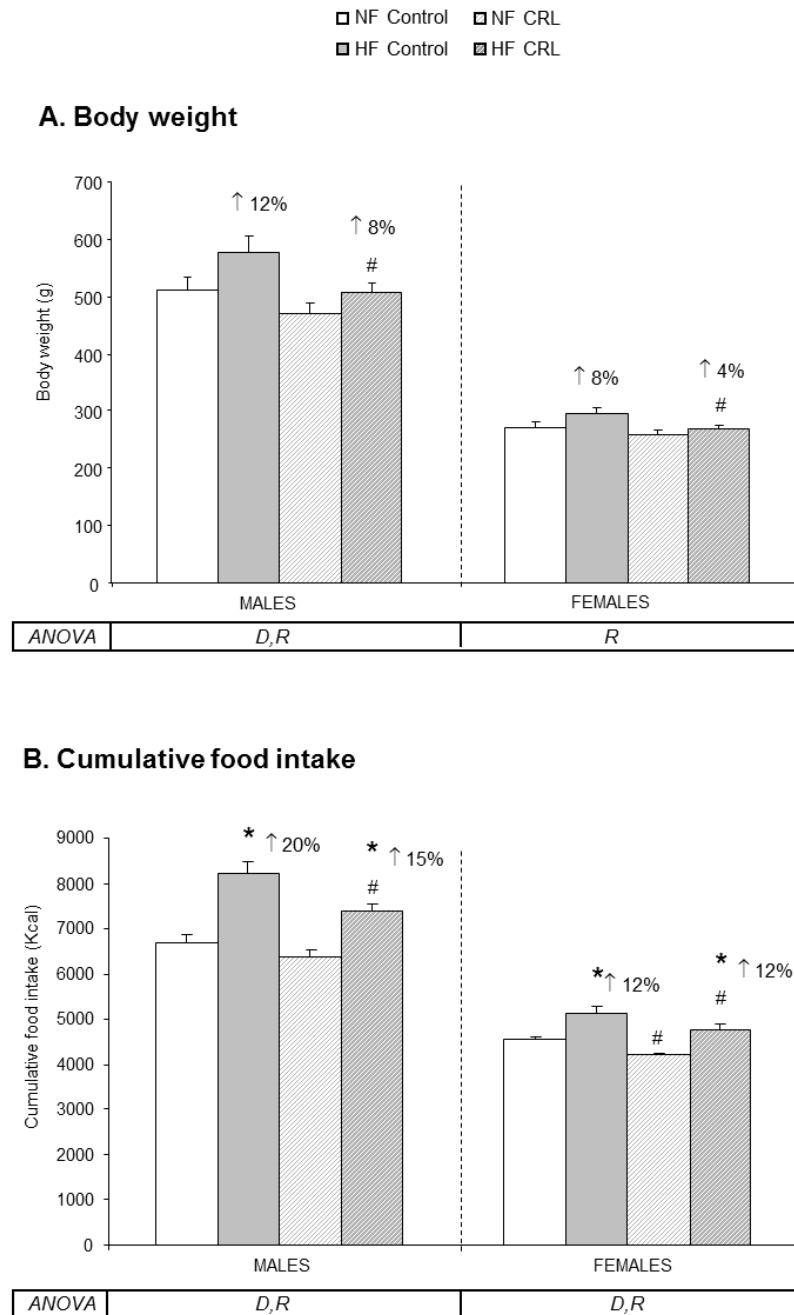


Figure 1. Body weight (A), and cumulative food intake from day 105 until day 200 (B) of male and female offspring of controls and caloric restricted dams during lactation (CRL) that were fed after day 105 with a NF or a HF diet until the age of 28 weeks. Results are expressed as the mean \pm SEM of 6 to 12 animals per group. STATISTICS: D, effect of the type of diet; R, effect of caloric restriction during lactation; and DxR, interaction between caloric restriction and diet (two-way ANOVA). *, effect of HF diet (Student's *t* test). #, effect of maternal caloric restriction (Student's *t* test). The percentages in brackets represent the increase as a consequence of the HF diet.

Table 1. Weights of inguinal, retroperitoneal, mesenteric and gonadal white adipose tissue of male and female offspring of control and caloric restricted dams during lactation (CRL), at the age of 28 weeks, under NF- and HF-diet feeding and under *ad libitum* feeding conditions.

		MALES		FEMALES	
		NF diet	HF diet	NF diet	HF diet
iWAT (g)	Control	11.5 ± 1.4	19.9 ± 2.6 *	3.12 ± 0.19	5.28 ± 0.50 *
	CRL	8.34 ± 0.94 #	12.7 ± 0.9 #*	2.87 ± 0.39	3.76 ± 0.31 #
	ANOVA		D, R		D, R
rWAT (g)	Control	14.5 ± 2.5	26.6 ± 3.9 *	2.78 ± 0.28	4.90 ± 0.81 *
	CRL	10.9 ± 1.5	18.5 ± 3.0 *	2.90 ± 0.32	4.42 ± 0.61 *
	ANOVA		D		D
mWAT (g)	Control	6.63 ± 1.09	11.4 ± 2.4 *	2.82 ± 0.36	4.36 ± 0.78
	CRL	4.64 ± 0.76	6.06 ± 0.52 #	2.03 ± 0.18 #	2.78 ± 0.30 #*
	ANOVA		D, R		D, R
gWAT (g)	Control	15.1 ± 2.4	22.9 ± 4.9	8.77 ± 0.95	14.4 ± 2.2 *
	CRL	12.2 ± 1.7	14.4 ± 2.1	7.09 ± 0.93	9.48 ± 1.27 #
	ANOVA				D, R
Adiposity	Control	9.08 ± 1.07	13.5 ± 1.2 *	6.27 ± 0.39	9.62 ± 1.09 *
Index (AI)	CRL	7.52 ± 0.84	10.3 ± 1.2 #*	5.73 ± 0.48	7.57 ± 0.65 *
	ANOVA		D, R		D

Weights are expressed in grams. Data are means ± SEM of 6 animals per group. STATISTICS: D, effect of the type of diet; and R, effect of caloric restriction during lactation (two-way ANOVA). *, NF vs. HF diet; #, Control vs. CRL (Student's t test). Abbreviations: inguinal white adipose tissue (iWAT), retroperitoneal white adipose tissue (rWAT), mesenteric white adipose tissue (mWAT), gonadal white adipose tissue (gWAT) and adiposity index (AI).

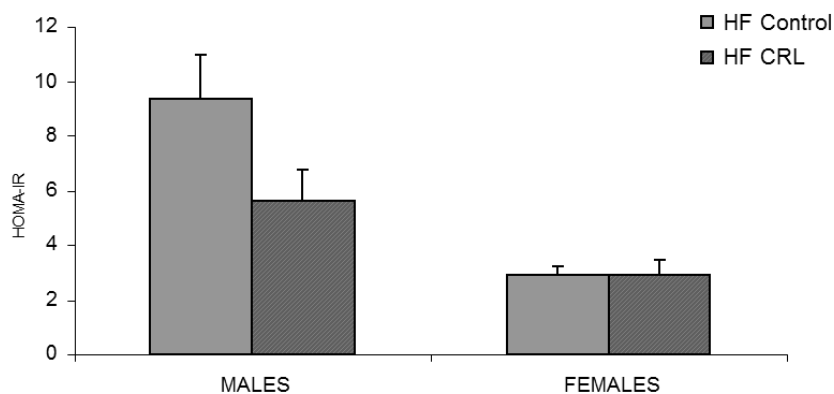


Figure 2. HOMA-IR index at the age of 28 weeks of male and female offspring of controls and maternal caloric restricted dams during lactation (CRL), which were fed with HF diet. Results are expressed as the mean ± SEM of 6 animals per group.

Table 2. Plasma glucose, insulin, leptin and triglyceride concentration under NF and HF diet under feeding conditions, and also for the latter under fasting conditions of male and female offspring of controls and caloric restricted dams during lactation (CRL) at the age of 28 weeks.

		MALES			FEMALES		
		Feeding		Fasting	Feeding		Fasting
		NF diet	HF diet	HF diet	NF diet	HF diet	HF diet
Glucose (mg/dl)	Control	104 ± 6	111 ± 2	94 ± 5 ⁺	106 ± 3	104 ± 5	89 ± 4 ⁺
	CRL	108 ± 5	104 ± 3	95 ± 4 ⁺	113 ± 2	114 ± 7	90 ± 5 ⁺
		ANOVA					
Insulin (µg/l)	Control	2.87 ± 0.72	6.01 ± 0.32 [*]	1.71 ± 0.27 ⁺	1.52 ± 0.29	1.34 ± 0.310	0.55 ± 0.04 ⁺
	CRL	2.15 ± 0.36	1.71 ± 0.24 [#]	0.97 ± 0.20 ⁺	1.14 ± 0.23	1.11 ± 0.21	0.55 ± 0.10 ⁺
		ANOVA		DxR			
Leptin (µg/l)	Control	7.68 ± 1.38	17.9 ± 1.4 [*]	8.98 ± 1.70 ⁺	2.33 ± 0.21	4.03 ± 0.65 [*]	2.80 ± 0.64
	CRL	6.21 ± 1.39	9.48 ± 1.46 [#]	5.31 ± 1.17 ⁺	2.13 ± 0.44	2.23 ± 0.38 [#]	1.00 ± 0.16 ^{+ #}
		ANOVA		DxR		R	
Triglycerides (mg/ml)	Control	2.81 ± 0.54	2.76 ± 0.40	2.47 ± 0.51 ⁺	2.87 ± 0.66	1.91 ± 0.39	1.18 ± 0.16
	CRL	2.76 ± 0.50	1.46 ± 0.35	0.902 ± 0.207 ⁺	2.77 ± 0.50	1.00 ± 0.16 [*]	0.500 ± 0.115 ^{+ #}
		ANOVA				D	

Results are expressed as the mean ± SEM of 6 animals per group. STATISTICS: D, effect of the type of diet; R, effect of caloric restriction during lactation; DxR, interaction between caloric restriction and diet (two-way ANOVA). *, NF vs. HF diet; #, Control vs. CRL⁺; *ad libitum* vs. Fasting (Student's t test)

Both male and female control rats, but not CRL rats, increased their plasma leptin levels as an effect of HF diet (two-way ANOVA). In addition, CRL females showed lower leptin concentration under both NF- and HF diet than their controls (two-way ANOVA). Male animals and CRL females, but not control females, decreased their circulating leptin levels after 12-h fasting (Student's t test); in fact, under HF diet conditions, CRL females had lower leptin levels than their controls both under feeding and fasting conditions (Student's t test).

Regarding TG, no differences were observed between control and CRL rats or after HF diet feeding in males (two-way ANOVA). A decrease in TG levels was found in both control and CRL male rats exposed to HF diet as an effect of fasting. However, unlike males, control and CRL females displayed lower TG concentration under HF diet compared with NF diet conditions (two-way ANOVA), but differences were more marked and significant by Student's t test only in CRL animals. Under HF diet conditions, TG levels decreased in CRL females, but not control females, as an effect of fasting, and levels in fasted CRL were lower than in fasted control animals (Student's t test).

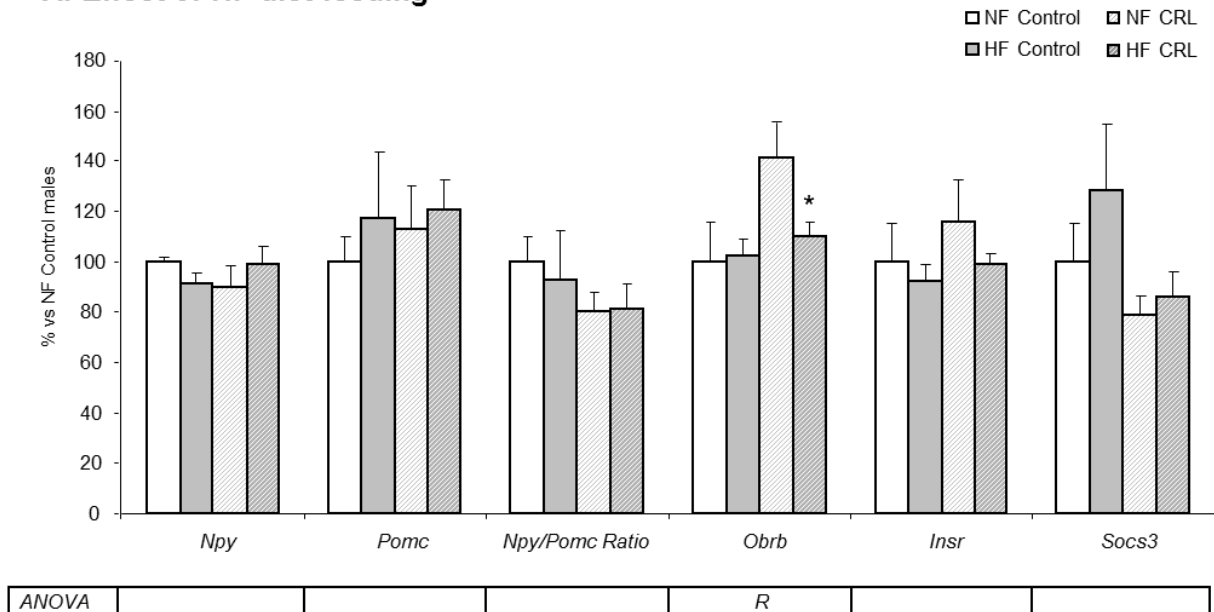
Hypothalamic mRNA levels of selected genes involved in energy balance in control and CRL male and female rats

Figure 3 shows mRNA expression levels of selected genes in the hypothalamus of male rats, under NF and HF diet under feeding conditions, and also for the latter under fasting conditions. No significant differences were found concerning *Npy* and *Pomc* mRNA levels as an effect of caloric restriction or HF diet exposure, either in the ratio *Npy/Pomc* (two-way ANOVA) (Fig 3A). Neither were any differences found concerning *Insr* mRNA levels. However, CRL animals showed higher *Obrb* mRNA levels than their controls (two-way ANOVA) (although these levels were lower by Student's t test under HF diet compared with NF diet), and a tendency to lower *Socs3* expression levels ($p=0.07$, two-way ANOVA).

HF-diet exposed male animals showed no significant changes in the expression levels of the analysed genes as an effect of fasting, with the exception of *Npy*, whose expression levels increased in control and CRL animals (two-way ANOVA) (Fig 3B), and of *Insr*, whose expression levels decreased only in control animals. Interestingly, the resulting *Npy/Pomc* mRNA ratio increased in CRL male rats after 12-h fasting, compared with fed conditions (Student's t test).

Hypothalamus - males

A. Effect of HF diet feeding



B. Effect of fasting in HF diet fed animals

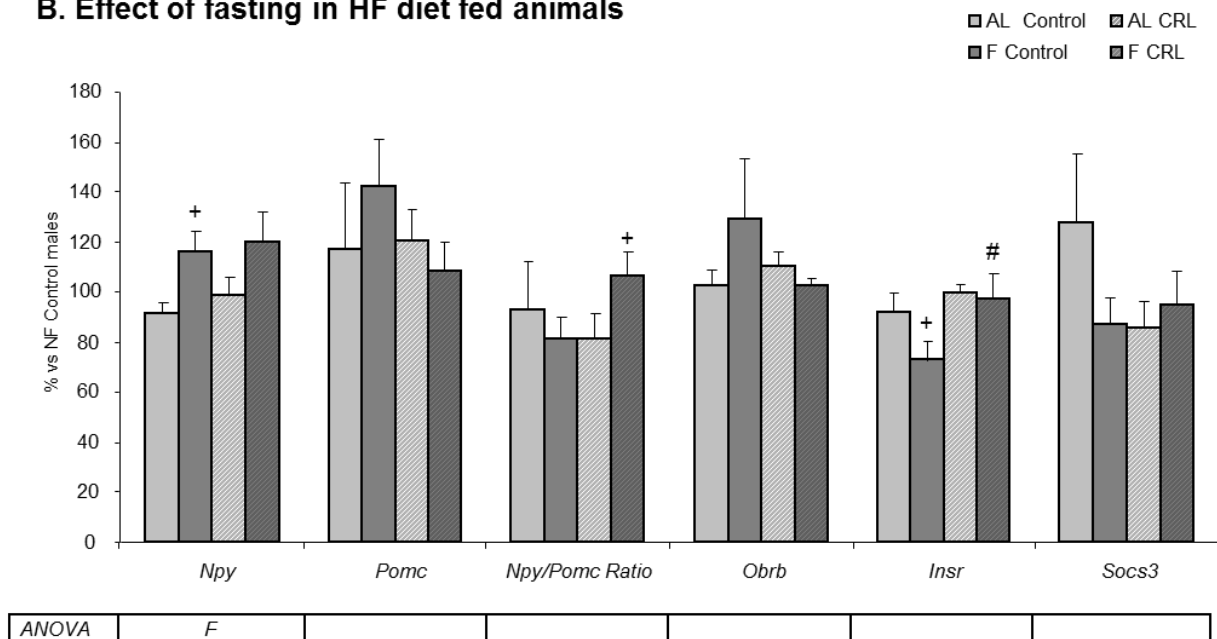


Figure 3. mRNA expression levels of neuropeptide Y (NPY), proopiomelanocortin (POMC), long form leptin receptor (ObRb), insulin receptor (InsR), and suppressor of cytokine signalling-3 (SOCS-3) and the NPY/POMC ratio in the hypothalamus of male rats, under NF and HF diet under feeding conditions (A), and also for the latter under fasting conditions (B). mRNA levels were measured by Real-time PCR and expressed as a percentage of the mean value of NF diet fed control males under *ad libitum* feeding conditions. Data are means \pm SEM (n = 6). STATISTICS: R, effect of maternal caloric restriction during lactation; F, effect of fasting (two-way ANOVA). *, NF vs. HF diet; #, Control vs. CR; +, *ad libitum* vs. Fasting (Student's t test).

Results on gene expression in the hypothalamus of females are shown in Figure 4. Control females increased *Npy* expression levels under HF diet, while CRL rats showed a decreased expression (interaction between caloric restriction and the type of diet, two-way ANOVA) (Fig 4A); no significant differences appeared in the expression of *Pomc* as an effect of caloric restriction or HF diet feeding (two-way ANOVA). The resulting *Npy/Pomc* mRNA ratio was lower in CRL female animals under HF diet compared with levels under NF diet, without significant changes in control animals. In addition, CRL female rats showed higher expression levels of *Insr* than their controls (two-way ANOVA), and a tendency to higher *Obrb* mRNA levels ($p=0.07$; two-way ANOVA). *SOCS-3* mRNA levels were higher in control and CRL animals under HF diet compared with levels under NF diet, without differences between control and CRL animals (two-way ANOVA).

Interestingly, under fasting conditions, HF-diet exposed CRL female animals increased *Npy* mRNA levels and the *Npy/Pomc* mRNA ratio; however, the expression levels of this gene and the *Npy/Pomc* mRNA ratio did not change in control animals as an effect of fasting (Student's t test) (Fig 4B). Furthermore, *Socs3* mRNA levels decreased in control animals as an effect of fasting, but remained unaltered in CRL female animals (Student's t test).

Retropertoneal WAT mRNA levels of selected genes involved in energy homeostasis in control and CRL male and female rats

Figure 5 shows mRNA expression levels of selected genes in WAT of male rats, under NF and HF diet under feeding conditions, and also for the latter under fasting conditions. Control male rats showed lower mRNA expression levels of *Ppar γ 2*, *Acc1*, *Gpat*, LDL, *Cpt1m* and *Insr* under HF diet, compared with those under NF diet (Student's t test), whereas this decrease was not found (for *Ppar γ 2*, *Cpt1m* and *Insr*) or was not significant (for *Acc1* and *Gpat*) in CRL animals (Student's t test) (Fig 5A). Moreover, *Gpat* mRNA levels were also significantly lower in CRL animals compared with their controls (two-way ANOVA), but were significant by Student's t test only under NF diet. *Glut4* expression levels decreased under HF diet in both control and CRL male rats, although the decrease was more marked and significant by Student's t test in CRL animals. Notably, when HF diet fed animals were deprived of food (Fig 5B), *Glut4* expression also decreased under fasting conditions (two-way ANOVA), but the decrease was significant by Student's t test only in CRL animals. However, *Gpat* mRNA levels decreased by Student's t test only in control rats, and remained unchanged in CRL animals. No response to fasting was found for the other genes studied, either in control or in CRL animals exposed to HF diet.

Hypothalamus - females

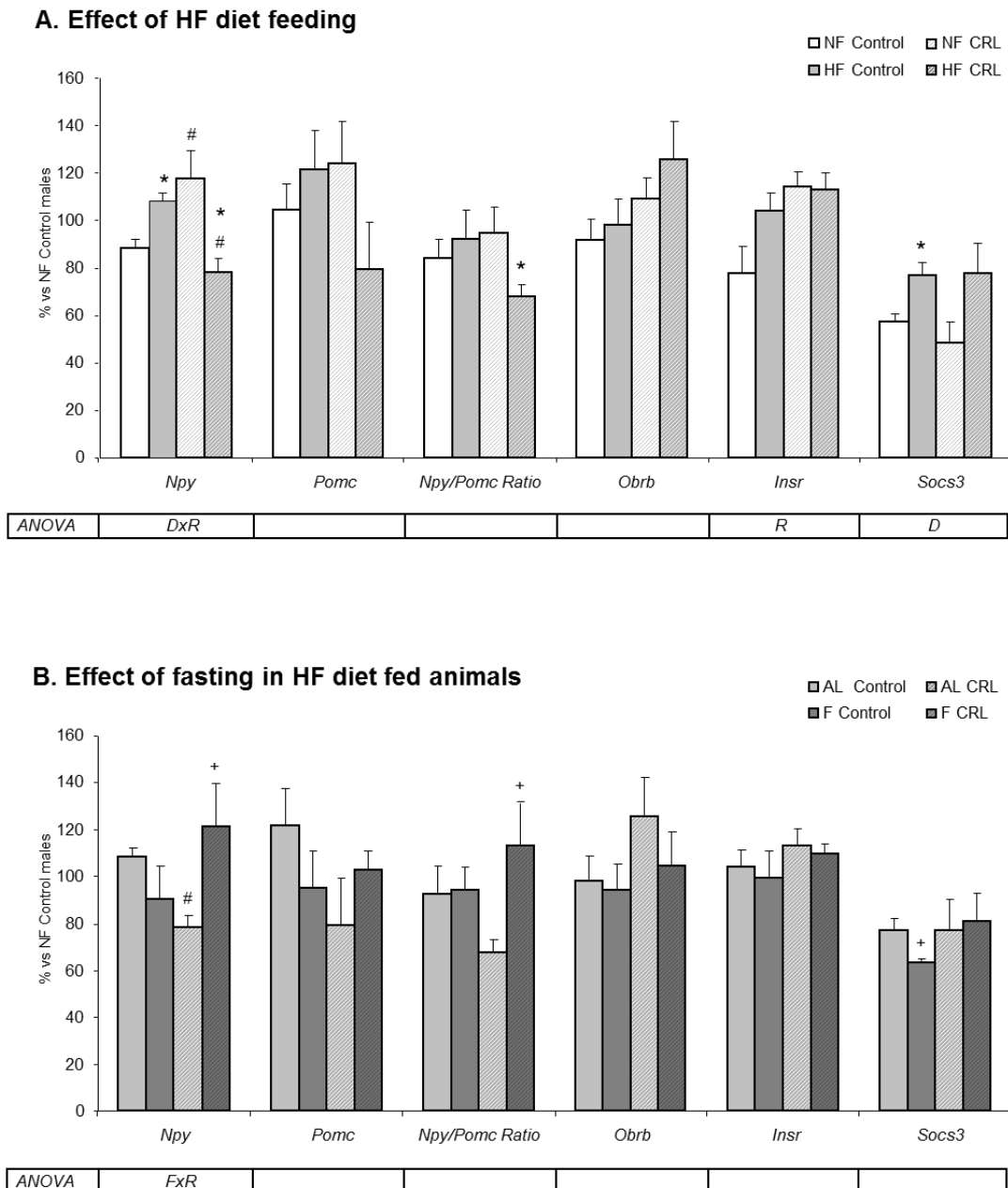
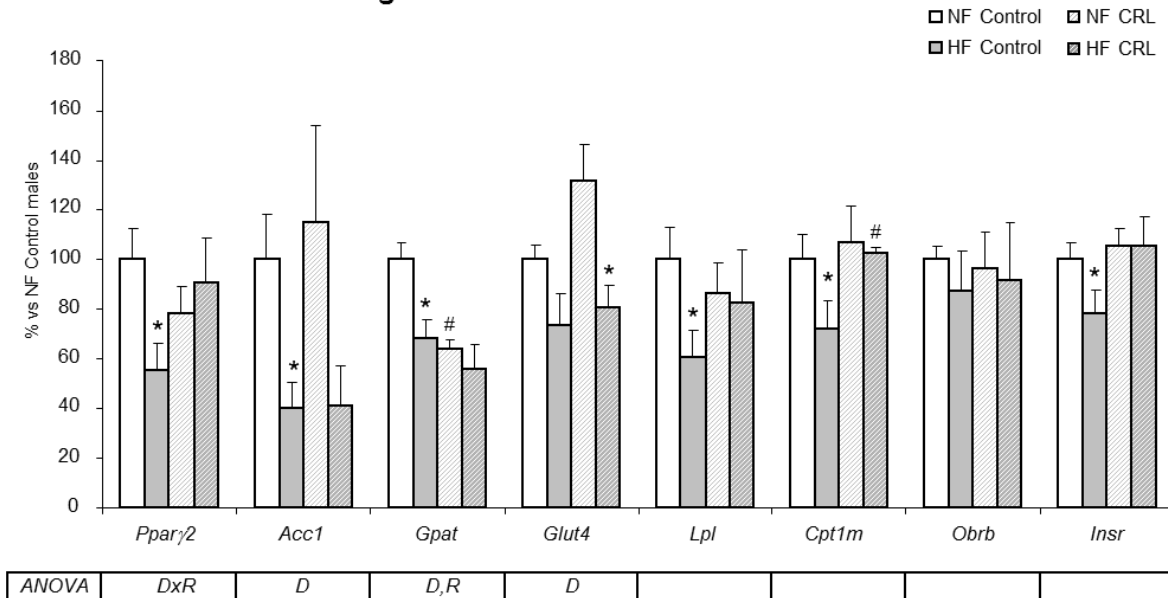


Figure 4. mRNA expression levels of neuropeptide Y (*Npy*), proopiomelanocortin (*Pomc*), long form leptin receptor (*Oubr*), insulin receptor (*Insr*), and suppressor of cytokine signaling 3 (*Soc3*) and the *Npy/Pomc* ratio in the hypothalamus of female rats, under NF and HF diet under feeding conditions (**A**), and also for the latter under fasting conditions (**B**). mRNA levels were measured by Real-time PCR and expressed as a percentage of the mean value of NF diet fed control males under *ad libitum* feeding conditions. Data are mean \pm SEM ($n = 6$). STATISTICS: D, effect of the type of diet; R, effect of caloric restriction during lactation; F, effect of feeding conditions; DxR, interaction between maternal caloric restriction and diet; and FxR, interaction between maternal caloric restriction and feeding conditions (two-way ANOVA). *, NF vs. HF diet; #, Control vs. CRL; +, *ad libitum* vs. Fasting (Student's *t* test).

WAT - males

A. Effect of HF diet feeding



B. Effect of fasting in HF diet fed animals

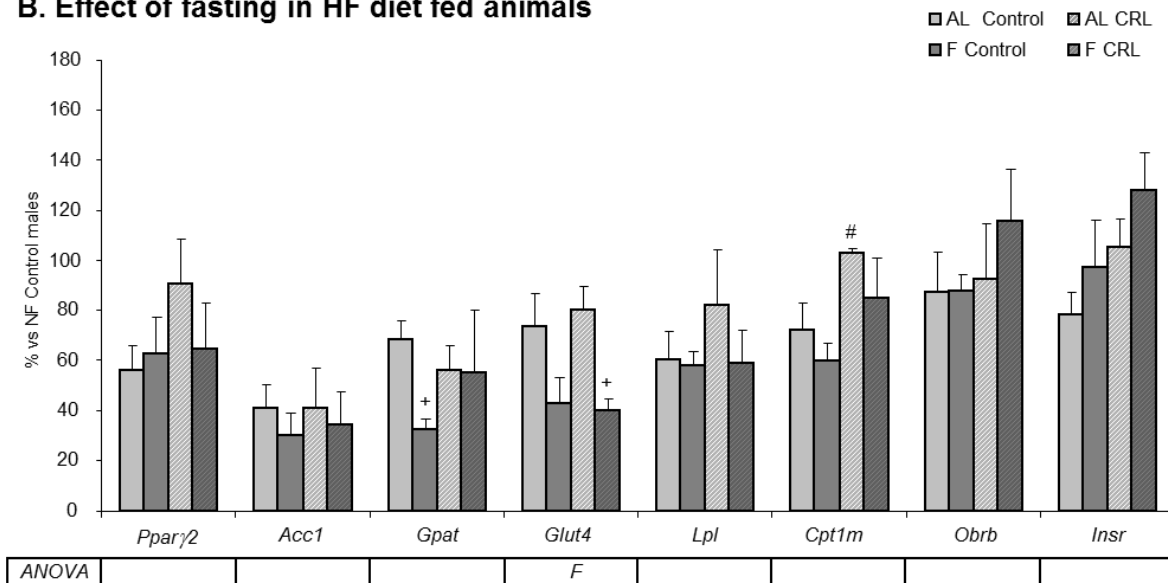
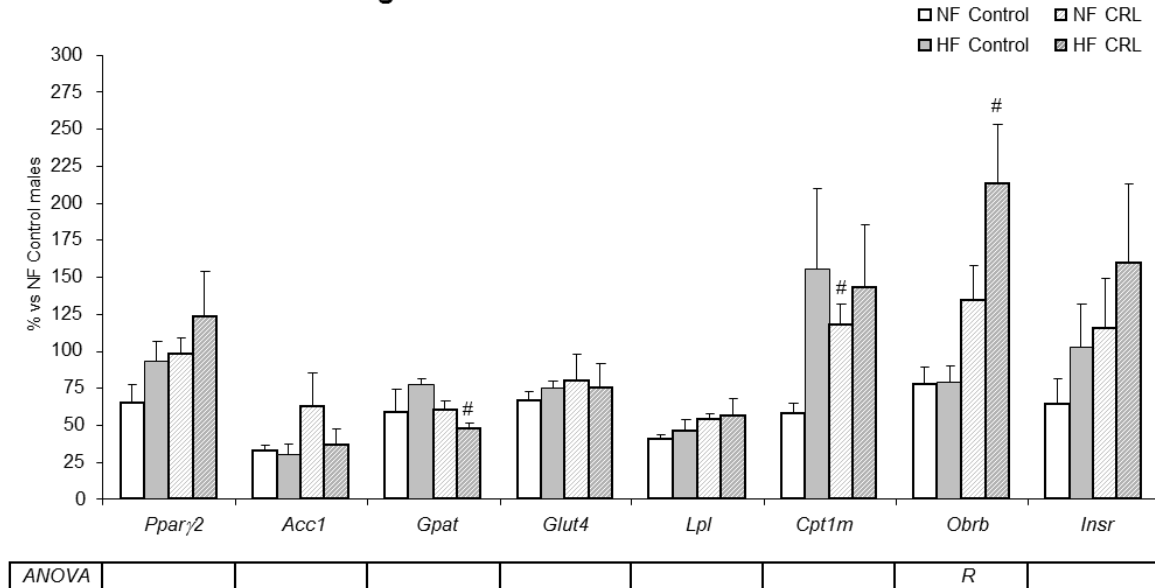


Figure 5. mRNA expression levels of selected genes in the WAT of male rats, under NF and HF diet under feeding conditions (A), and also for the latter under fasting conditions (B). mRNA levels were measured by Real-time PCR and expressed as a percentage of the mean value of NF diet fed control males under *ad libitum* feeding conditions. Data are mean \pm SEM (n = 6). Genes determined were: peroxisome proliferator activated receptor gamma 2 (*Pparγ2*), acetyl-coenzyme A carboxylase alpha (*Acc1*), glycerol-3-phosphate acyltransferase (*Gpat*), glucose transporter 4 (*Glut4*), lipoprotein lipase (*Lpl*), muscle carnitine palmitoyltransferase 1a (*Cpt1m*), the leptin receptor (*Obrb*) and the insulin receptor (*Insr*). STATISTICS: D, effect of the type of diet; R, effect of caloric restriction during lactation; F, effect of feeding conditions; DxR, interaction between maternal caloric restriction and diet; and FxR, interaction between maternal caloric restriction and feeding conditions (two-way ANOVA). *, NF vs. HF diet; #, Control vs. CRL; +, *ad libitum* vs. Fasting (Student's t test).

Results on gene expression in WAT of females are shown in Figure 6.

WAT - females

A. Effect of HF diet feeding



B. Effect of fasting in HF diet fed animals

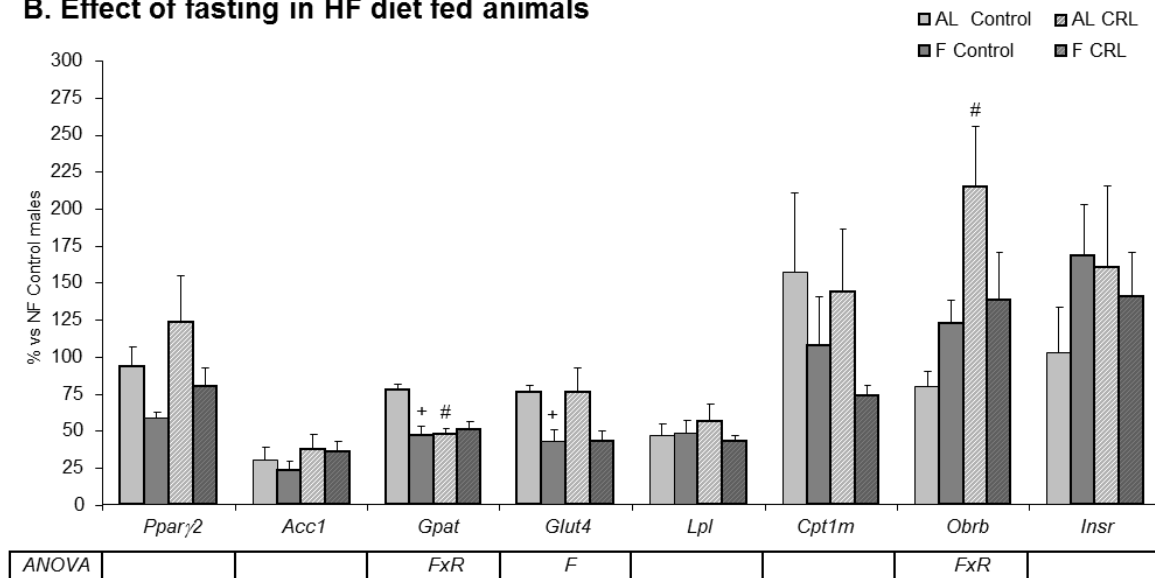


Figure 6. mRNA expression levels of selected genes in the WAT of female rats, under NF and HF diet under feeding conditions (A), and also for the latter under fasting conditions (B). mRNA levels were measured by Real-time PCR and expressed as a percentage of the mean value of NF diet fed control males under *ad libitum* feeding conditions. Data are mean \pm SEM (n = 6). Genes determined were: peroxisome proliferator activated receptor gamma 2 (*Pparγ2*), acetyl-coenzyme A carboxylase alpha (*Acc1*), glycerol-3-phosphate acyltransferase (*Gpat*), glucose transporter 4 (*Glut4*), lipoprotein lipase (*Lpl*), muscle carnitine palmitoyltransferase 1a (CPT1m), the leptin receptor (*Obrb*) and the insulin receptor (*Insr*). STATISTICS: R, effect of caloric restriction during lactation; F, effect of feeding conditions; FxR, interaction between maternal caloric restriction and feeding conditions (two-way ANOVA). *, NF vs. HF diet; #, Control vs. CRL⁺; *ad libitum* vs. Fasting (Student's t test).

No significant differences were found in females as an effect of HF diet or caloric restriction in the expression of *Ppar γ 2*, *Acc1*, *Glut4*, *Lpl* and *Insr* in WAT (two-way ANOVA) (Figure 6A). CRL females showed higher *Obrb* mRNA levels than their controls (two-way ANOVA), greater *Cpt1m* mRNA levels but only under NF diet (Student's t test), and lower mRNA levels on *Gpat* but only under HF diet (Student's t test).

CRL female animals under HF diet, besides expressing greater *Obrb* mRNA levels than their controls under this diet, they showed a different response to fasting conditions compared with their controls (interaction between caloric restriction and fasting, two-way ANOVA): *Obrb* mRNA levels tended to decrease in CRL animals, but to increase in controls (Figure 6B). In turn, *Gpat* mRNA levels decreased after fasting in control rats without showing significant changes in CRL (interaction between caloric restriction and fasting, two-way ANOVA). Both control and CRL females exposed to HF diet decreased their *Glut4* mRNA levels with fasting (two-way ANOVA), while mRNA levels of *Acc1*, *Lpl*, *Cpt1m* and *Insr* did not change under fasting conditions in either control or CRL females (two-way ANOVA).

Discussion

It is well known that maternal nutrition during perinatal periods may program the development of their offspring towards different consequences (Bertram and Hanson 2001; Cripps, et al. 2005; Gluckman and Hanson 2004; McMillen, et al. 2005; Taylor and Poston 2007). We previously described that a moderate caloric restriction (30%) in lactating dams confers certain protection against obesity development and from related metabolic alterations in adult life, particularly insulin resistance and hyperleptinemia (Palou, et al. 2010b). However, little is known about the mechanisms that underlie these alterations and whether they are associated with changes in the expression of key genes involved in energy homeostasis. Here, we show that programming of gene expression related with central and peripheral leptin and insulin sensibility may be involved in the beneficial effects of moderate caloric restriction during lactation.

In accordance with our previous results in the same cohort of animals at younger ages (Palou, et al. 2010b), we also show here that the offspring of caloric restricted dams during the suckling period display lower body weight and lower fat content in adulthood, compared with their controls, the difference being more patent when animals are exposed to the challenge of HF diet feeding. The lower body weight can be explained, at least in part, by lower food intake. Comparison of accumulated calories eaten in adulthood during the 13-week period of exposure to NF or HF diet showed that food intake was lower in CRL animals compared with their controls, both under NF and HF diet. The increased intake of calories associated with HF diet exposure was lower in CRL male animals compared with their controls (increase of 15% and 20%, respectively), but was similar in CRL female rats compared with their controls (increase of 12% and 12%, respectively). Remmers et al. (Remmers, et al. 2008c) also showed reduced body weight, fat content and food intake in male and female caloric restricted rats during lactation obtained by large litter size; in contrast, malnutrition produced by protein restriction to the dams during lactation has been associated in the adult offspring with reduced body weight, despite no changes in food intake (de Moura, et al. 2007; de Souza Caldeira Filho and Moura 2000; Fagundes, et al. 2007).

Central resistance to insulin and/or leptin have been proposed as important mechanisms responsible for the dysregulation of energy homeostasis, which may lead to obesity (Esteghamati, et al. 2009; Levin and Dunn-Meynell 2002; Lustig, et al. 2004). Regarding insulin, male rats are known to have a higher tendency for hyperinsulinemia induced by hyperlipidic diets than females (Priego, et al. 2008). In this sense, we have previously described that male offspring of caloric restricted rats during lactation are protected against diet-induced hyperinsulinemia and insulin resistance when animals are exposed for 5 weeks in adulthood to HF diet (Palou, et al. 2010b); in agreement, here we also found lower insulin levels under feeding conditions and a tendency to lower HOMA-IR in CRL males exposed for a longer period (13 weeks) to HF diet, suggesting a better resistance of CRL male rats against the detrimental effects of an obesogenic environment on circulating insulin profile. Concerning the leptin system, CRL animals showed lower circulating leptin levels, particularly under HF diet feeding, compared with controls. This is in accordance with their lower body weight and agrees with the results obtained when these animals were younger (20 weeks old) (Palou, et al. 2010b). Interestingly, lower leptin levels have been associated with a better sensitivity to leptin (Sánchez, et al. 2008) but, in contrast, higher circulating leptin levels may contribute to the energy imbalance induced by HF diet feeding or age and are involved with the impairment of the fasting-induced suppression of leptin production (Iossa, et al. 1999; Pico, et al. 2007; Sánchez, et al. 2008). On the other hand, gender-differences have been described concerning the leptin system; female rats seem to be relatively more sensitive to leptin system regulation, whereas male rats are more sensitive to insulin regulation (Clegg, et al. 2003; Priego, et al. 2008; Woods, et al. 2003). Nevertheless, HF diet fed control male animals showed decreased plasma leptin levels under fasting conditions, whereas the decrease was not significant in female control animals. However, both HF diet fed male and female CRL animals responded to fasting conditions by lowering their plasma leptin levels.

Leptin and insulin are thought to regulate feeding behavior through their abilities to modulate the transcription of several neuropeptide genes. Thus, to ascertain whether changes in food intake between control and CRL animals could be related with central effects of these hormones, the expression of hypothalamic genes related with leptin and insulin action were determined. NPY is considered one of the main peptides regulating food intake with orexigenic activity (Dryden, et al. 1994). Increased NPY protein and/or mRNA levels have been described in the hypothalamus of different animal models of obesity (Dryden, et al. 1994); for instance, higher *Npy* mRNA levels have been found in female rats chronically fed with HF diet (Priego, et al. 2009b). We also found here that *Npy* mRNA levels increased in control female animals when exposed to HF diet, but, interestingly, they decreased in CRL females exposed to this diet; moreover HF diet exposed CRL females, but not their controls, displayed higher *Npy* mRNA levels after fasting conditions; this suggests not only a better adaptation to this hyperlipidic diet, but also a better response to the feeding/fasting patterns. However, it is worth mentioning that, under NF diet, CRL female animals displayed higher *Npy* mRNA levels than their controls; this fact cannot explain the hypophagia or the lower body weight of these animals compared with their controls. This suggests that caloric restriction may also affect other processes that could counteract the increased expression of *Npy* in these animals. Unlike females, *Npy* mRNA expression levels did not change in male animals, either as an effect of diet or caloric restriction.

POMC is the precursor of the anorexigenic peptide alpha-melanocyte stimulating hormone (α MSH) (Bell, et al. 2000), but no significant differences were found between control and CRL animals concerning *Pomc* expression. Considering that both NPY and POMC are the main neuropeptides stimulating and inhibiting food intake, respectively, it is interesting to highlight that the *Npy/Pomc* ratio decreased in CRL females under HF diet and increased under fasting

conditions in both HF diet exposed CRL male and female animals, but not in controls, although the difference in males did not reach statistical significance ($p=0.06$, Student's *t* test). These results may also contribute to explain the better control of food intake and body weight in CRL rats under HF diet.

This pattern of expression of neuropeptides involved in food intake control and regulated at the central level by leptin and insulin may be indicative of a better sensitivity to these hormones. The responsiveness of the hypothalamus to leptin and insulin action depends not only on the circulating levels of these hormones, but also on different factors determining the sensitivity to these signals, such as the leptin and insulin receptors or the cytokine inhibitory protein *Socs3* (Baskin, et al. 1998; Peiser, et al. 2000). The ObRb, the longest isoform of all leptin receptors, is mainly expressed in the hypothalamus and is considered to be the signaling-competent isoform (Baskin, et al. 1998). This form is sensitive to genetic and physiological interventions that change circulating leptin levels, indicating that overexpression of *Obrb* in the hypothalamus may contribute to increased leptin sensitivity (Baskin, et al. 1998). Here, we measured the expression of *Obrb* in the hypothalamus to evaluate possible differences in leptin responsiveness between control and CRL rats under NF or HF diet feeding. CRL male rats showed higher *Obrb* mRNA levels than controls, particularly under NF diet; a non significant tendency ($p=0.073$) was also observed in CRL females. This is likely to be, an indicator of better leptin sensitivity and a higher resistance to obesity development. On the other hand, SOCS3 is a leptin-inducible inhibitor of leptin signaling, and a potential mediator of leptin resistance in obesity (Bjorbaek, et al. 1998). No significant differences were found between control and CRL animals concerning the expression of levels of *Socs3* in the hypothalamus, but, notably, CRL males presented a tendency ($p=0.074$) to lower *Socs3* expression levels both under NF (21% reduction) and HF diet (45% reduction). This reduction could contribute to increase leptin action and attenuate sensitivity to diet-induced obesity. This is supported by the fact that mice, which are heterozygous for an *Socs3* gene deletion and hence have a 50% reduction of *Socs3* expression, exhibit enhanced *Obrb* activation induced by leptin (Howard, et al. 2004). In addition, SOCS3 is also an inhibitor of insulin action (Emanuelli, et al. 2000). Thus, these changes in the expression levels of *Socs3* in the hypothalamus of CRL male animals could also account for a better responsiveness to the central action of insulin. Insulin, like leptin, is secreted in proportion to fat stores, and also enters the CNS in proportion to its plasma levels (Plum, et al. 2006). Insulin receptors are expressed in neurons of brain areas involved in feeding control, and central administration of insulin can reduce food intake and body weight (Morton and Schwartz 2001; Plum, et al. 2006). Interestingly, CRL female animals showed increased *Insr* mRNA levels with respect to their controls which may determine a better response to insulin action, improving their feeding behaviour. All in all, these results may illustrate that a better responsiveness to the central action of leptin (in CRL male animals) and insulin (in CRL female animals) could be the mechanisms underlying the better control of food intake and body weight at the central level.

In addition to the hypothalamus, the adipose tissue is a target of the peripheral action of leptin and insulin. The adaptations of this tissue to an obesogenic environment and also to feeding conditions are another main determinant of the propensity to suffer obesity or other metabolic alterations (Palou, et al. 2008; Priego, et al. 2008). In this sense, visceral fat accumulation, rather than subcutaneous, has been strongly linked to features of the metabolic syndrome, including leptin resistance, type 2 diabetes, hypertension and dyslipidemia (Despres 2001; Masuzaki, et al. 2001; Weiss 2007). Here, we show that CRL rats appear to be more prepared to resist obesity development, the protective effect being more evident in males. This can be associated in male animals with an improvement of adipose tissue responsiveness to insulin. In fact, CRL male animals were resistant to the decrease occurring in *Insr* mRNA levels in the

internal (retroperitoneal) WAT depot under HF diet feeding. In addition, CRL males had a better response to HF diet feeding, by maintaining fat uptake capacity and its storage by the adipose tissue, as well as the fatty acid oxidation capacity, whereas their controls presented an impairment of these processes. In fact, control male rats showed a decreased expression of lipogenic-related genes such as *Ppar γ 2*, *Lpl*, which are regulated by this transcription factor (Tsai and Maeda 2005), *Acc1* and *Gpat*, and also of the catabolic-related gene *Cpt1m* after HF diet feeding, whereas expression levels were mostly maintained unaltered in CRL male animals (with the exception of *Acc1*, which showed a non significant tendency to decrease). A greater capacity to channel the excess of energy from the diet to the adipose tissue has been related with a better adaptive response to a HF diet (Priego, et al. 2008) and higher sensitivity to insulin (Slawik and Vidal-Puig 2006). In fact, insulin regulates *Ppar γ* mRNA expression by the adipose tissue (Rieusset, et al. 1999), and this factor is involved in whole-body insulin sensitivity, probably through its effects on adipocyte metabolism and secretory function (Tsai and Maeda 2005). Thus, these results agree with an impairment of insulin sensitivity of control males under HF diet, at least at the gene expression level, whereas insulin sensitivity was not apparently impaired in CRL male animals under HF diet. This is also in accordance with the pattern observed in the HOMA-IR in these animals. Increased insulin sensitivity has also been previously described in the offspring of severe protein and caloric restricted rats during lactation (Martin-Gronert, et al. 2008; Palou, et al. 2010b; Sampaio de Freitas, et al. 2003; Zambrano, et al. 2006). Other conditions during the early neonatal period, such as the supplement of suckling rats with physiological doses of leptin has also been associated in male animals with improved insulin sensitivity (Sánchez, et al. 2008) and with better metabolic adaptations to HF diet feeding (Priego, et al. 2010); in concrete, changes at the gene expression level in the adipose tissue evidenced a better capacity of leptin-treated animals to handle and partition the excess of fuel under HF diet feeding, preventing other metabolic disorders related with HF-diet feeding, such as hepatic lipid accumulation (Priego, et al. 2010).

Unlike males, no significant differences were found between control and CRL female animals concerning the expression of *Insr* and of metabolism-related genes in the adipose tissue, although it must be mentioned that female animals appear to be more protected against the detrimental effects of HF diet on the expression of these genes, as previously described (Priego, et al. 2008). These results also point out the increased capacity of the adipose tissue of female animals to store an excess of fat (Priego, et al. 2008), in accordance with their healthier response to obesogenic environments in comparison with males (Priego, et al. 2009a; Priego, et al. 2008; Woods, et al. 2003). These results are also in accordance with the lack of apparent effects of caloric restriction during lactation on insulin resistance (measured by the HOMA-IR) in CRL female animals under HF diet.

It is worth noting, HF diet exposed CRL females presented lower circulating fasting TG levels than their controls; thus, although they did not apparently improve their insulin sensitivity, like CRL males, a better blood TG profile could be related to an improvement of the capacity to store the excess of fuel in the adipose tissue. Results of Zammit (Zammit 2002) have demonstrated that repeated exposure of the liver to elevated levels of insulin has a potent stimulatory effect on hepatic TG production. Thus, this over-stimulation of hepatic TG production through insulin action may outline a mechanistic basis for the development of leptin resistance, even independently of HF diet feeding (Krechowec, et al. 2006). Elevated fasting plasma TG levels have also been described in the offspring of 30% caloric restricted dams throughout pregnancy (Krechowec, et al. 2006), and this has been found in conjunction with hyperinsulinemia and leptin resistance. In this sense, female CRL rats displayed higher *Obrb* mRNA levels in the adipose tissue than their controls, and, when exposed to HF diet, their expression levels were sensitive to feeding/fasting conditions, which is also in accordance with

the circulating leptin patterns observed. Interestingly, CRL female animals also showed higher expression levels of CPT1 in the adipose tissue than their controls, although only under NF diet. This suggests that CRL female rats may have greater fatty acid oxidation capacity in adipose tissue, which is of great importance for the control of whole body weight and fat reserves, and may be related to the pattern of *Obrb* expression in this tissue, as previously described (Priego, et al. 2010). All in all, these results may indicate that CRL female animals are more sensitive to the peripheral action of leptin, and this agrees with the lower plasma leptin levels and the better response of the circulating hormone to fasting, suggesting that CRL females are more resistant to the development of overweight under HF diet conditions than their controls.

In conclusion, we show here that moderate caloric restriction (30%) in lactating dams results in lower body weight, adiposity and food intake in their male and female offspring in adulthood, but the mechanisms underlying these adaptations are gender-dependent. Changes in blood hormone concentration and at the gene expression level suggest that CRL male rats seem to be more protected against HF-diet induced peripheral insulin resistance, and this results in an improved capacity of the adipose tissue to handle and store the excess of fuel from the diet. In addition, these animals also show improved capacity to respond to leptin at the central level. CRL female animals, in turn, appear to be programmed for a better sensitivity to the peripheral actions of leptin on the adipose tissue and to the central action of insulin. Both mechanisms may have similar outcomes in males and females, providing a better adaptation to the challenge of HF diet feeding. These results could help our understanding of the differential regulation of energy homeostasis in both genders, as well as the mechanisms responsible for the beneficial effects of moderate caloric restriction of the dams during lactation.

Manuscript

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

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J Nutr Biochem. 2014;25(2):208-18

Title page

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

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Running title: Early biomarkers of health

Statement of financial support: We gratefully acknowledge the Spanish Government (grants AGL2009-11277, AGL2012-33692), the European Union (BIOCLAIMS FP7-244995), and the Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red Fisiopatología de la Obesidad y Nutrición, CIBERobn. Our Laboratory is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, EU Contract: n° FP6-506360). Jadwiga Konieczna is granted with a PhD fellowship entitled "beca para la formación de personal investigador, en el marco de un programa operativo cofinanciado por el Fondo Social Europeo".

Conflict of interest: None

Abstract

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

Key words: adipose tissue, calorie restriction, early markers, lactation, liver, obesity

Introduction

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

Besides, although direct animal-to-human extrapolation is complex, regarding the beneficial effects of moderate calorie restriction during lactation on offspring metabolic health in rats, it would be of interest to consider its potential application in humans. However, studies on early postnatal undernutrition in lactating women are fewer due to ethical implications. Epidemiologic reports, and especially those related to reduction of maternal food intake during lactation, have described the effects on infant body weight gain or on maternal milk production depending on the severity of the restriction (Dewey 1998b; Dusdieker, et al. 1994). Other studies carried out in baboons, have shown no significant effect on milk output with a moderate calorie restriction of 20%, but milk output was significantly lower in those with 40% calorie restriction (Roberts, et al. 1985). Therefore, although 30% calorie restriction during lactation in rats has shown benefits in the offspring preventing later obesity (Palou, et al. 2012; Palou, et al. 2010b), this restriction might be associated with a reduction in milk production and hence does not seem to be feasible to extrapolate to humans, if applicable.

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

whether these changes in gene expression levels at early stages were maintained in adulthood, evaluating their behavior under stressful dietary conditions, such as HF-diet feeding, and their potential relation with the improvement of metabolic health.

Methods and Materials

Animals and Experimental Design

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

The study was conducted on male and female *Wistar* rats from 16 different litters following the protocol as is described below.

Animals were housed under standard conditions, that is, controlled temperature (22°C), a 12h light-dark cycle, free access to tap water. Briefly, 16 virgin female *Wistar* rats weighing between 225g and 260g were mated with male rats (Charles River Laboratories, Barcelona, Spain). After matching, each female was placed in an individual cage. At day 1 after delivery, pups were weighed and the size of all litters was adjusted to 10 neonates per mother (five males and five females, when possible) to ensure adequate and standardized nutrition until weaning. Dams were assigned to either control (n=11 dams) or calorie restricted (n=5 dams) group. Throughout the lactation period, starting on day 1 after delivery until weaning (day 21), control dams were fed *ad libitum* with standard chow diet (3 kcal/g, with 8% calories from fat; Panlab, Barcelona, Spain), while calorie restricted dams were fed daily with 20% calorie restricted diet. Calorie restriction was performed by offering each dam a daily amount of food corresponding to 80% of the calories that they should eat according to their body weight. This amount was calculated considering the calories consumed daily by their control animals under *ad libitum* feeding conditions. During the lactating period, body weight of male and female offspring of control and calorie restricted dams (control and CRL, respectively) was followed.

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

Another set of animals (n=11-16 per group) were weaned at 21 days of life, and were then housed 2 per cage (paired with another animal from the same group), and fed on a normal-fat (NF) standard chow diet (3.8 kcal/g, with 10% calories from fat; Research Diets, NJ, ScholarOne, 375 Greenbrier Drive, Charlottesville, VA, 22901 USA) until the age of 4 months. Then, male and female rats from both control and CRL-groups were distributed into two groups: one group (n= 6-8 animals per group) that continued with NF-diet, and another group (n=5-8 animals per group) that was exposed to HF-diet (4.7 kcal/g, with 45% calories from fat; Research Diets, Inc., NJ, USA) until the age of 6 months. HF-diet contained 5.5% calories from soybean oil and 39.5% from lard. Body weight and food intake of the offspring were recorded from weaning until the age of 6 months, when animals were killed. Body length (from

the tip of the nose to the anus) and body composition (by EchoMRI-700TM, Echo Medical Systems, LLC., TX, USA) were measured without anesthesia when animals were 21 days old and 6 months old.

Two weeks before sacrifice, blood samples of control and CRL-animals were collected under *ad libitum* feeding conditions and after 12 h fasting. Plasma was obtained as described above to analyze circulating parameters. Finally, at the age of 6 months, NF- and HF-diet fed rats were killed under *ad libitum* feeding conditions by decapitation during the first 2 h at the beginning of the light cycle and on different consecutive days (including animals from each group every day). At killing, different WAT depots (gWAT, iWAT, mWAT and rWAT) and liver were rapidly removed, weighed, frozen in liquid nitrogen and stored at -80°C until RNA analysis.

Measurement of circulating parameters and hepatic lipid content

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

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

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

Oral Fat Tolerance Test (OFTT)

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

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

RNA extraction

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

Real-time quantitative PCR-analysis

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

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

Statistical analysis

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

Results

Phenotypic traits and food intake

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

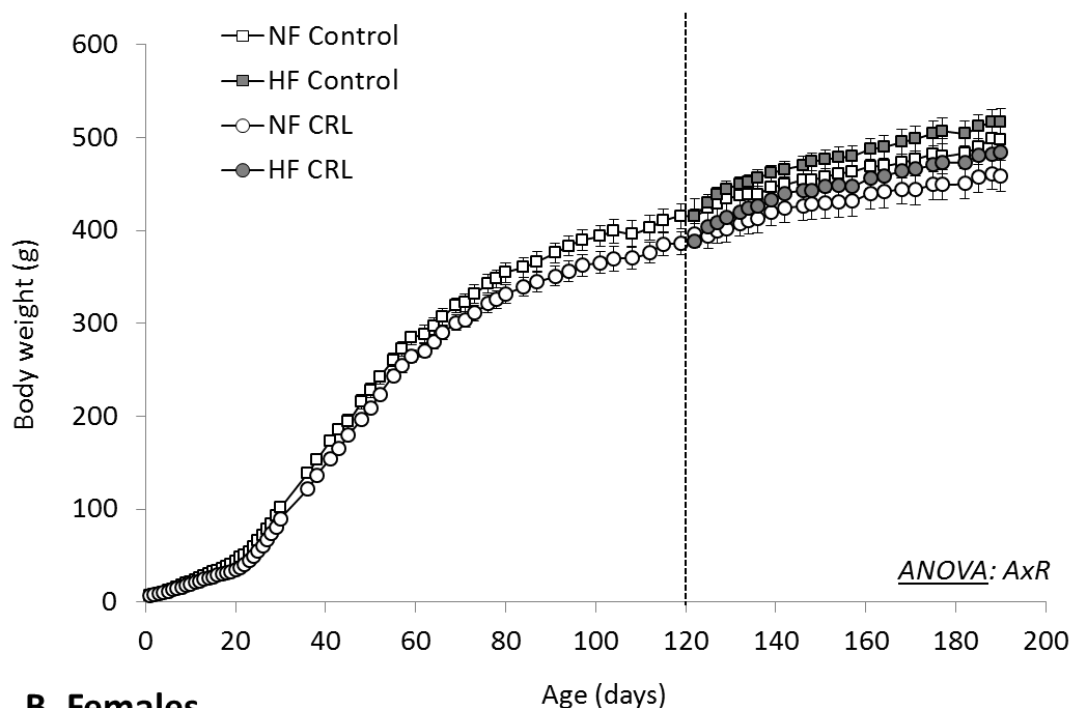
The lower body weight of CRL-animals could be due to shorter body length, for this reason we measured the length from the tip of the nose to the anus (body length) at different ages (Table 1). At weaning (21 days), both male and female CRL-pups showed a significant shorter body length than their controls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

At weaning, CRL-animals showed higher body lean content and lower body fat content than controls; they also showed a decrease in weight of the main (gonadal, inguinal, mesenteric and retroperitoneal) WAT depots ($p < 0.05$, two-way ANOVA) (Table 1). At the age of 6 months, differences concerning body fat content between control and CRL-animals were found under HF-diet ($p = 0.067$, two-way ANOVA), particularly in females ($p < 0.05$, Student's *t* test). A similar trend was found when analyzing the weight of different WAT depots. Concerning body lean content, HF-diet fed CRL-animals showed significantly higher lean content than controls ($p < 0.05$, two-way ANOVA). Under NF-diet, only CRL-females, but not males, showed a higher percentage of lean mass and a tendency to lower fat content (interactive effect between sex and calorie restriction, $p < 0.05$, two-way ANOVA). The same tendency was seen for the weight of main WAT depots, with the exception of the gonadal one, which showed a significant decrease in both male and female CRL-animals ($p < 0.05$, two-way ANOVA).

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

Body weight over time

A. Males



B. Females

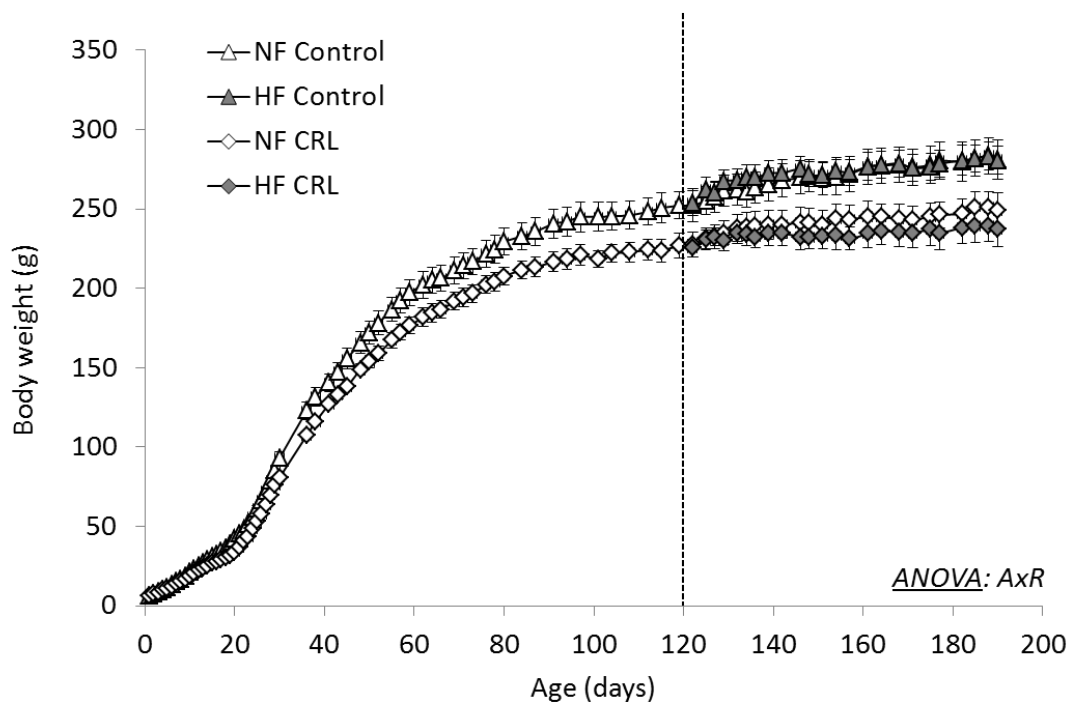


Figure 1. Body weight over time of male and female offspring of control and calorie restricted dams during lactation (CRL) from day 1 until day 190 of age. From the age of 120 days, half of the animals of both control and CRL-group were exposed to a HF-diet, and the other half remained with NF-diet.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

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

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

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

Oral fat tolerance test

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

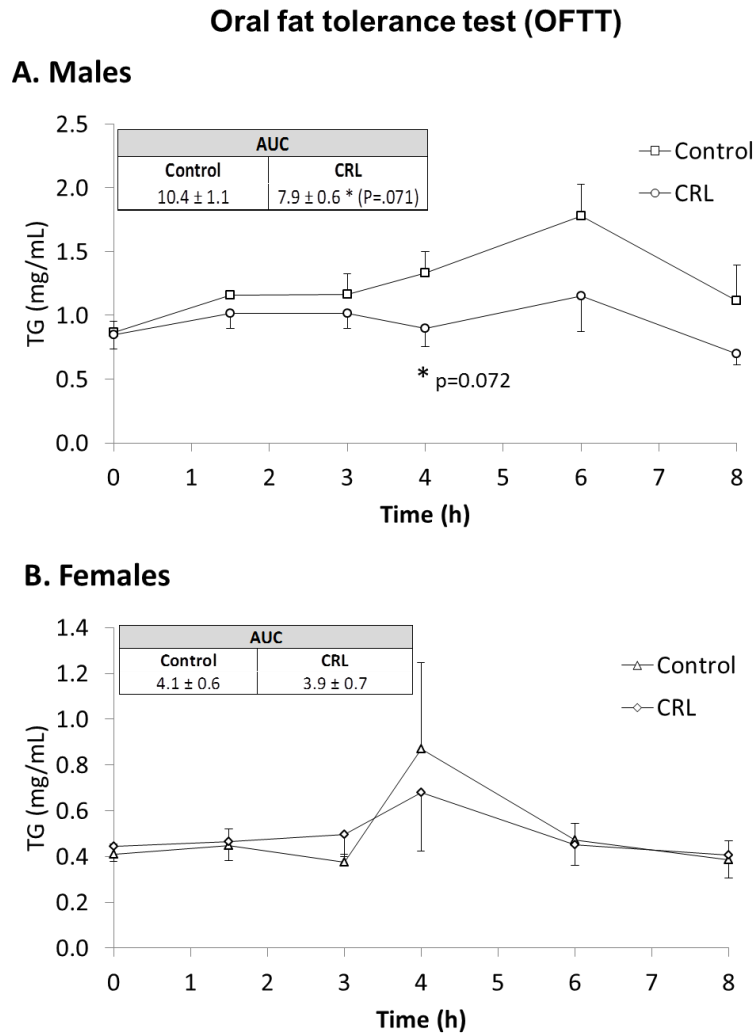


Figure 2. Triglyceride (TG) response (mg/mL) to an oral fat tolerance test (OFTT) in male (A) and female (B) offspring of control and calorie restricted dams during lactation (CRL) at 4 months of age. Data are mean \pm S.E.M. (n= 6 animals per group). Statistics: *, CRL vs Control ($p<0.05$, Student's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

Circulating parameters

Circulating glucose, insulin, leptin, adiponectin, NEFA, and TG levels of control and CRL-animals at the age of 21 days and 6 months are shown in Table 2. At weaning, compared to controls, male and female CRL-pups displayed lower circulating levels of glucose, insulin and leptin ($p<0.05$, two-way ANOVA) and higher adiponectin levels (only CRL-males, $p<0.05$ Student's *t* test). CRL-pups also showed lower leptin-to-adiponectin (L/A) ratio than controls

($p < 0.05$, Student's t test). No differences were found concerning NEFA and TG levels between control and CRL-pups.

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

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

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

At the age of 6 months, under NF-diet conditions, no significant differences were found in glucose levels between control and CRL-rats, although fasted CRL-females showed lower glucose levels than controls ($p < 0.05$, Student's t test). Notably, only CRL-animals showed a significant decrease in glucose levels as an effect of fasting conditions ($p < 0.05$, Paired t test). CRL-animals, and particularly females, showed lower levels of insulin than their controls ($p < 0.05$, ANOVA repeated measures), and CRL-females also showed lower leptin and L/A ratio ($p < 0.05$, Student's t test; interactive effect between calorie restriction and sex, $p < 0.05$, two-way ANOVA). We did not find significant differences between control and CRL-animals concerning HOMA index under NF-diet, but female CRL-animals showed a tendency to lower values than controls ($p = 0.076$, Student's t test). No significant differences were found in adiponectin, NEFA or TG levels due to maternal calorie restriction in NF-diet fed rats. When exposed to HF-diet, a sex- and treatment- dependent response to feeding conditions was found in glucose levels (interactive effect between sex, calorie restriction and feeding conditions, $p < 0.05$, ANOVA repeated measures); this is explained by the lower fasted glucose levels of CRL-females, but not males, with respect to their controls ($p < 0.05$, Student's t test) and with respect to the fed state ($p < 0.05$, Paired t test). Interestingly, CRL-animals showed lower leptin, L/A ratio and TG levels than control rats ($p < 0.05$, two-way ANOVA). No significant differences were found in insulin, adiponectin or NEFA levels as an effect of calorie restriction during lactation under a HF-diet. However, HF-diet exposed CRL-females, but not males, displayed significantly lower HOMA index than their controls ($p < 0.05$, Student's t test).

Hepatic lipid content

Figure 3 shows hepatic lipid content in the different groups of animals at the age of 6 months. CRL-animals presented lower lipid content compared to controls, both under NF- and HF-diet conditions ($p < 0.05$, Student's t test).

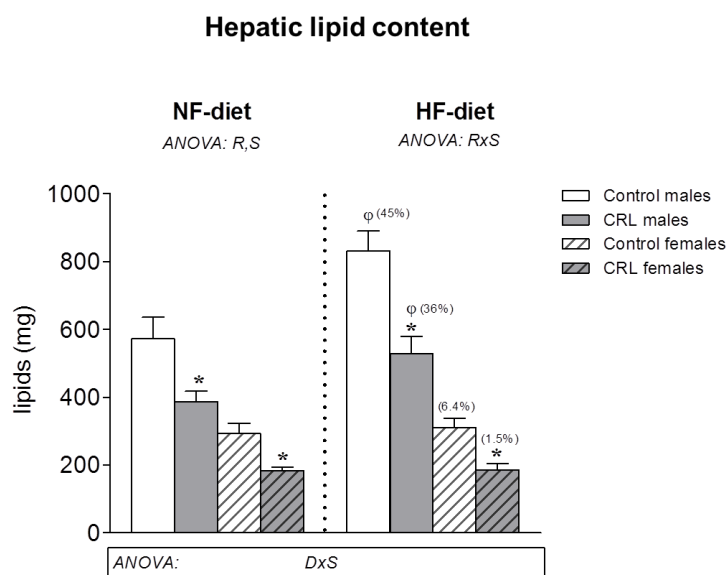


Figure 3. Hepatic lipid content (mg) in male and female offspring control and calorie restricted dams during lactation (CRL) at 6 months of age under NF- and HF-diet. The number in brackets indicates the percentage of lipid increment in liver as effect of HF-diet vs their respective controls under NF-diet.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

Interestingly, hepatic lipid content increased in male animals when exposed to HF-diet, but this increase was less marked in CRL-animals ($p < 0.05$, Student's *t* test). In contrast, HF-diet feeding, in the conditions of this study, did not induce significant changes in hepatic lipid content in females, either in control or CRL-animals.

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

Figure 4A shows mRNA expression levels of selected genes in rWAT of 21-day-old control and CRL-pups. The retroperitoneal depot was selected to be analyzed for gene expression, based on the literature showing that this depot seems to be more sensitive to nutritional status, compared with other depots (Palou, et al. 2010b). CRL-pups showed lower mRNA levels of *Fasn* and greater mRNA levels of *Ppara*, *Atgl*, *Insr* and *Irs1* than controls ($p < 0.05$, two-way ANOVA). In addition, an interaction between the effects of calorie restriction during lactation and sex was observed in the mRNA levels of *Gpat* ($p < 0.05$, two-way ANOVA), since CRL-female animals, but not males, showed a significant decrease in their expression levels ($p < 0.05$, Student's *t* test). No significant differences were observed in the expression levels of the other genes analyzed (*Pparγ*, *Cpt1b*, and *Obrb*) between control and CRL-animals. Figures 4B and 4C show mRNA levels of 6-month-old controls and CRL-animals in rWAT under NF- and HF-diet conditions, respectively.

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

Results on gene expression in liver of 21-day-old control and CRL-pups are shown in Figure 5A. CRL-animals showed lower mRNA levels of *Srebp1c* and higher mRNA levels of *Cpt1a* and *Obrb* compared to their controls ($p < 0.05$, two-way ANOVA). CRL-animals also showed *Atgl* mRNA levels slightly higher than controls ($p = 0.087$, two-way ANOVA). No significant differences were found concerning mRNA levels of *Fasn*, *Gpat*, *Ppara*, *Insr* or *Irs1* as an effect of calorie restriction during lactation. Figures 5B and 5C show mRNA expression levels in the liver of 6-month-old control and CRL-animals under NF- and under HF-diet, respectively. NF-diet fed CRL-animals showed lower mRNA levels of *Gpat* ($p < 0.05$, two-way ANOVA) and *Srebp1c* ($p = 0.072$, two-way ANOVA, specially CRL-males by Student's *t* test, $p < 0.05$) with respect to controls. No significant differences between control and CRL-animals were found for the rest of genes studied under NF-diet conditions, although an interaction between maternal calorie restriction during lactation and sex was found for mRNA levels of *Cpt1a*. When exposed to HF-diet, sex-dependent differences between control and CRL-animals were found. Concretely, no changes or a trend to lower mRNA levels of *Srebp1c*, *Ppara*, *Insr*, *Irs1* and *Obrb* were found in CRL-males with respect to their controls, whereas higher mRNA levels (*Irs1* and *Obrb*) or a tendency to higher levels (*Srebp1c*, *Ppara*, *Insr*) was found in females (interactive effect between calorie restriction and sex, $p < 0.05$, two-way ANOVA).

mRNA expression in rWAT

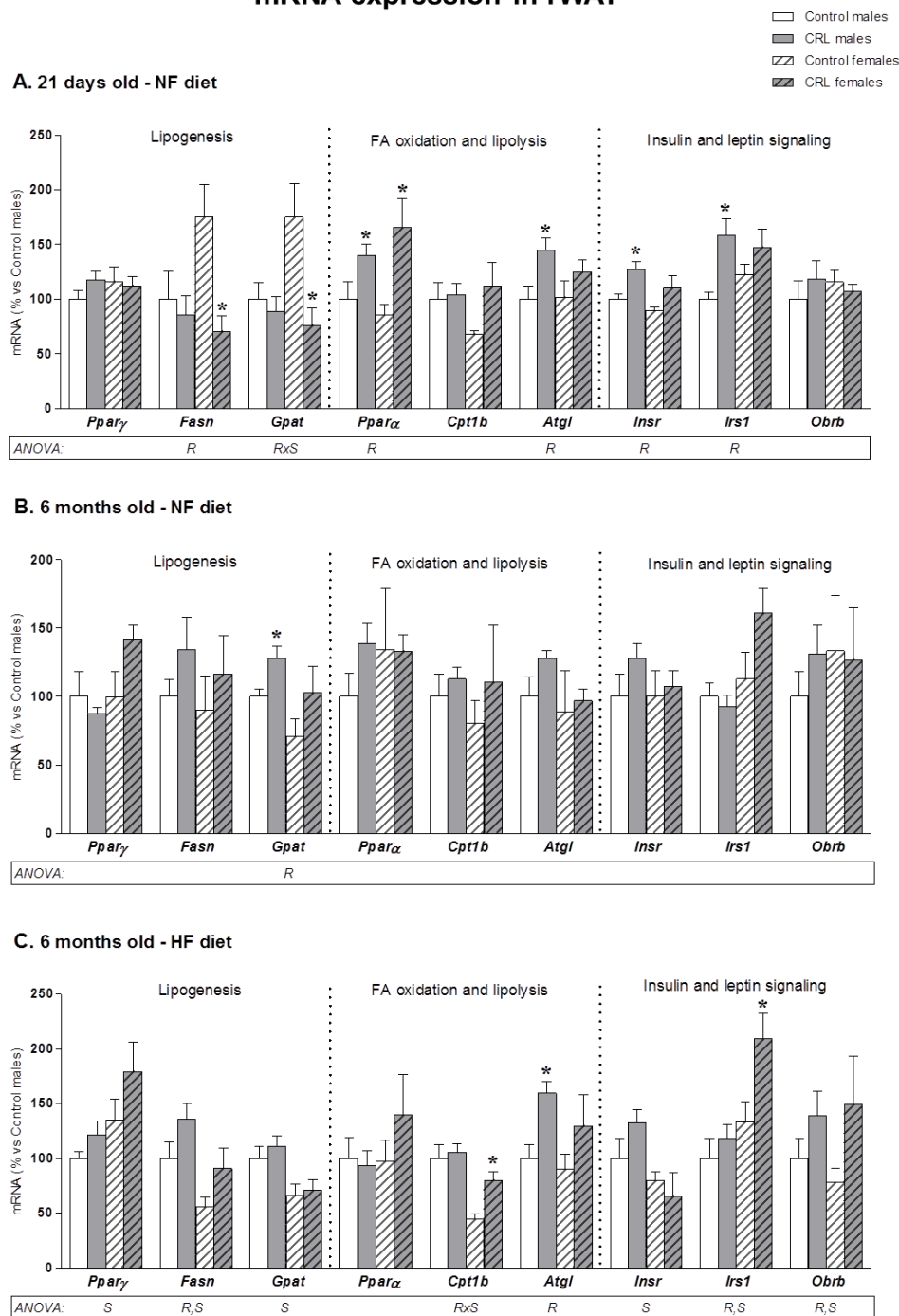


Figure 4. mRNA levels of energy balance and lipid metabolism related genes in retroperitoneal white adipose tissue (rWAT) of male and female offspring of control and calorie restricted dams during lactation (CRL), at 21 days old (A), 6 months old under NF-diet (B), and 6 months old under HF-diet (C).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

mRNA expression in liver

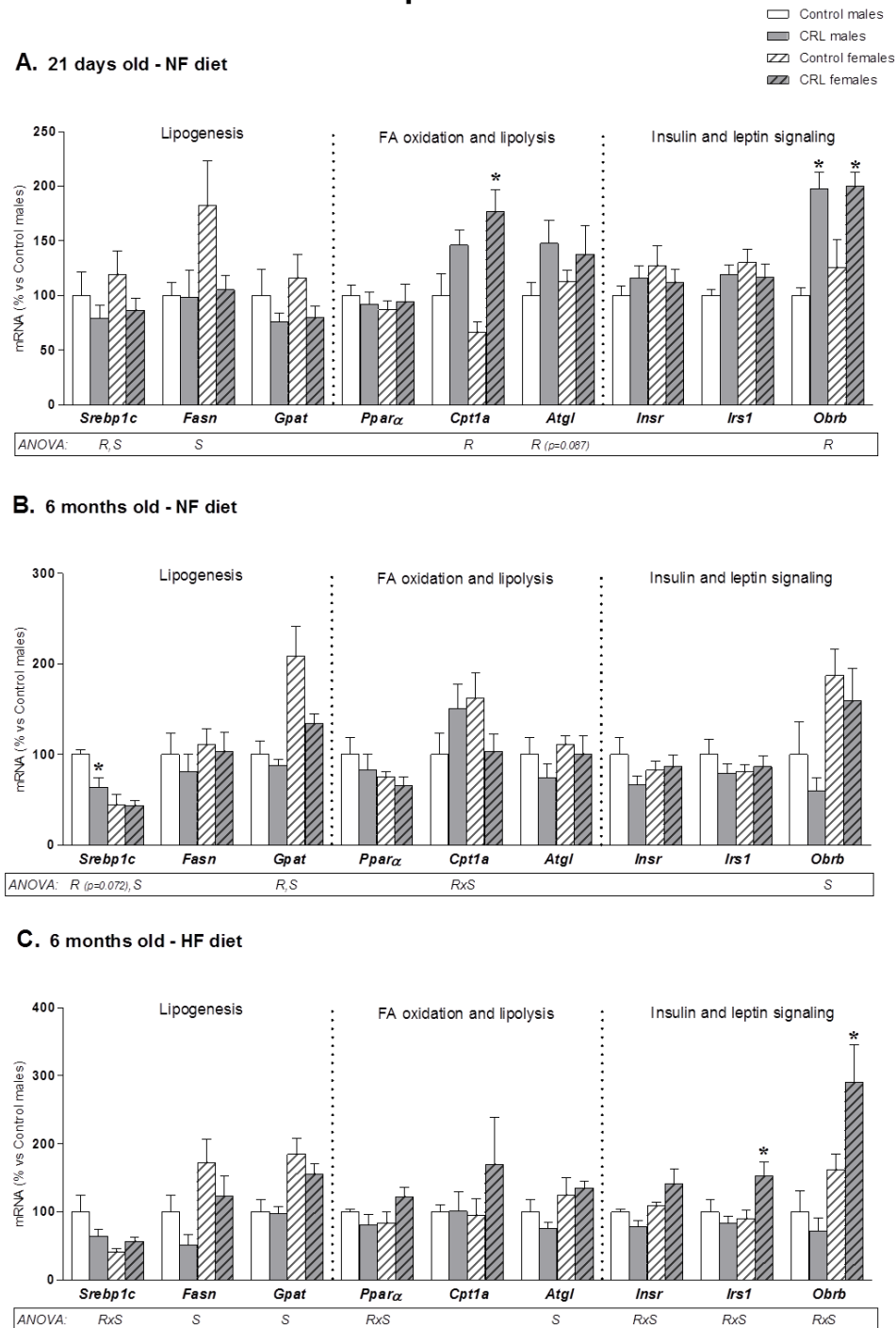


Figure 5. mRNA levels of energy balance and lipid metabolism related genes in liver of male and female offspring of control and calorie restricted dams during lactation (CRL), at 21 days old (A), 6 months old under NF-diet (B), and 6 months old under HF-diet (C). mRNA levels were measured by RT-qPCR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

Correlation analysis of selected parameters

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

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

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

Correlations were performed for all genes analyzed, but only those showing significant correlations are indicated. r, Pearson correlation index; p, p-values for Pearson correlation coefficient. Abbreviations: *ad libitum* (AL), insulin resistance HOMA index (HOMA-IR), insulin receptor substrate 1 (*Irs1*), long form leptin receptor (*Obrb*), triglycerides (TG), retroperitoneal white adipose tissue (rWAT), sterol regulatory element-binding protein 1c (*Srebp1c*). Statistics: *, p-value<0.05; **, p-value<0.005.

Discussion

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

Regarding the characterization of the model, in short, we found that the offspring of 20% calorie restricted dams during lactation showed lower body weight in adulthood, as well as lower adiposity when exposed to HF-diet conditions, with the differences being more evident

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

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

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

Circulating lipid profile could also give some clues to characterize the metabolic health of these animals in relation with lipid metabolism and handling. In this sense, we previously described that 30% moderate maternal calorie restriction in lactating rats resulted in an improved capacity of the adipose tissue of male adult offspring to handle and store excess fuel when exposed to HF-diet, as evidenced by changes at the gene expression level of key genes involved in lipid metabolism (Palou, et al. 2011). Thus, to further study whether a more modest 20% calorie restriction during lactation was associated with a better capacity to handle dietary lipids and improved lipemia, an OFTT was performed at the age of 4 months. Impaired postprandial metabolism of TG in terms of a higher peak or delayed clearance has been associated with higher cardiovascular risk (Ansar, et al. 2011). Results showed a trend to a lower increase in circulating TG after the oral load of fat in CRL-male animals, compared to controls. It must be noted that, although differences between both groups were slight, this

improvement in CRL-males was already evident at the age of 4 months, and prior to apparent signs of an impaired function related to age. In female animals, differences between control and CRL were not evident, at least at this age. The protective effects of calorie restriction during lactation against dyslipidemia were further evident later on when animals were exposed to HF-diet, since CRL-animals, both males and females, showed lower TG levels than controls under this dietary stressor. Interestingly, CRL-animals, both under NF- and HF-diet, also displayed lower hepatic lipid content, the hallmark of nonalcoholic fatty liver disease (NAFLD), which is well recognized as being part of the metabolic syndrome (Postic and Girard 2008). In accordance with the literature (Priego, et al. 2008), fat overloading in the diet brings a different response in males and females, since males seems to be more prone to accumulating lipids in the liver. Here it is also shown that, in both sexes, the response to HF-diet seem to be better in CRL-animals compared to controls.

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

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

In addition, 21-day-old CRL-pups showed a better expression profile in rWAT of genes involved in lipid metabolism. In concrete, they showed higher expression levels of *Atgl*, the main protein involved in TG mobilization, and of *PPAR α* , the major transcription factor regulating fatty acid oxidation. In adulthood and under HF-diet feeding conditions, CRL-animals also maintained higher mRNA expression levels of *Atgl* than controls. Moreover, at 6 months old, HF-diet-fed CRL-females displayed higher expression levels of *Cpt1b*. Curiously,

CRL-females but not males, showed lower *Fasn* and *Gpat* mRNA levels than controls at weaning, but when animals were 6 months old, CRL-rats displayed higher mRNA expression levels of *Gpat*, under NF-diet conditions, and higher mRNA levels of *Fasn*, under HF-diet. The greater expression levels of these lipogenic genes in adult CRL-animals were not associated with any significant increase in the size of this adipose tissue depot, but could reflect increased capacity of these animals to drive and store excess energy to the adipose tissue. Therefore, differences in the expression levels of *Fasn* and *Gpat* between controls and CRL-animals seem to be dependent on the age, type of diet and sex of animals.

The liver plays an important role in energy metabolism and is also a target of the peripheral action of leptin and insulin. Concerning insulin signalling, HF-diet-fed female animals in the CRL-group, but not males, showed higher mRNA levels of *Irs1* compared with their controls, although no significant changes were previously observed at weaning. A similar trend, but not significant, was also found for *Insr*. Interestingly, 21-day-old CRL-pups showed higher *Obrb* mRNA expression levels, and this pattern was maintained in adult CRL-females under HF-diet. The physiological role of peripheral leptin signalling, particularly in liver, and its relative contribution to whole-body energy metabolism, still remain unclear. Peripheral leptin signalling has been considered dispensable for whole body energy homeostasis, since mice lacking peripheral *Obrb* expression showed no significant alterations in tissue and whole-body energy metabolism (Guo, et al. 2007). Nevertheless, leptin action in liver has been shown to attenuate hepatic glucose production and insulin resistance under normal conditions of lean animals, hence contributing to the inhibitory effects of insulin on gluconeogenesis (Brabant, et al. 2005). This effect was largely lost in obese animals, which showed decreased expression of the leptin receptor gene in liver (Brabant, et al. 2005). Hence, maintenance of higher *Obrb* expression levels occurring in CRL-females when fed a HF-diet could be related with increased peripheral leptin and insulin sensitivity, as evidenced by the presence of lower circulating levels of leptin, as well as lower L/A ratio and HOMA index. Interestingly, hepatic *Obrb* mRNA levels in adult animals were correlated negatively with hepatic lipids and with circulating levels of insulin under *ad libitum* feeding conditions (although the latter with a p value of only 0.076). All in all, these results suggest that increased *Obrb* mRNA levels in liver may be considered beneficial in terms of leptin-to-insulin signalling crosstalk and concerning hepatic lipid storage. Thus, *Obrb* mRNA levels in liver could be considered as a marker of metabolic health.

Other changes occurring at the gene expression level in liver are also in accordance with the protection of CRL-animals against hepatic TG accumulation. That is, CRL-pups showed lower mRNA expression levels of *Srebp1c* than controls. This trend was maintained in adult male animals, particularly under NF-diet conditions. Activation of SREBP1c has been related with increased lipogenesis and has been considered one of the molecular mediators of hepatic steatosis (Browning and Horton 2004). Interestingly, mRNA levels of *Srebp1c* in liver were positively correlated with hepatic lipid content and also with HOMA index in adult animals (the latter with p=0.070). In addition, adult CRL-animals under NF-diet also showed lower expression levels of *Gpat*, the rate limiting enzyme in TG synthesis (Wendel, et al. 2009). Moreover, at weaning, CRL-pups showed higher hepatic mRNA expression levels of genes related with lipid mobilization and fatty acid oxidation, *Atgl* and *Cpt1a*. ATGL is a key triacylglycerol lipase in the liver, and its overproduction has been associated with reduced steatosis and improved insulin signal transduction in this tissue (Turpin, et al. 2011). On the other hand, increased hepatic *Cpt1a* expression levels may also be a mechanism contributing to the protection of these animals against excessive body weight gain and related metabolic alterations, as evidenced in mice with adenovirus-mediated overexpression of *Cpt1a* in liver (Orellana-Gavaldà, et al. 2011). However, changes in the expression levels of the genes, *Atgl*

and *Cpt1a*, by the effect of calorie restriction during lactation, were no longer observed in adulthood.

In conclusion, a very modest maternal calorie restriction of 20% during lactation in rats programs the offspring for better metabolic health in terms of body weight and lipid handling. This condition during lactation determines early adaptations in rWAT and liver, affecting lipogenic and oxidative capacity and increasing their sensitivity to the peripheral effects of leptin and insulin, which suggests a better control of energy metabolism. These adaptations occurring in early ages are partially maintained in adulthood, and are particularly evident when animals are exposed to an obesogenic environment. Interestingly, among the genes that exhibit changes at the expression level in early ages, *Irs1* in rWAT and *Obrb* and *Srebp1c* in liver are of relevance because their transcript levels in adult animals were associated with lower hepatic lipid content and improved circulating parameters, thus they could be considered as potential biomarkers of a healthy phenotype. Validation of these biomarkers in human samples, particularly blood cells, which can be easily collected in sufficient quantities by a minimally invasive method, becomes of interest.

Manuscript

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

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Genes Nutr. 2014;9(1):366

Title page

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Statement of financial support: The research leading to these results was supported by the European Union's Seventh Framework Programme FP7 2007-2013 under grant agreement n. 244995 (BIOCLAIMS Project), Spanish Government (grants AGL2009-11277 and AGL2012-33692 to AP), and the Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red Fisiopatología de la Obesidad y Nutrición, CIBERobn. The Laboratory of Molecular Biology, Nutrition and Biotechnology (Nutrigenomics) belongs to the Nutrigenomics-group, awarded as "Group of Excellence" of CAIB and supported by "Direcció General d'Universitats, Recerca i Transferència del Coneixement" of Regional Government (CAIB) and FEDER funds (EU Contract: n. FP6-506360). Jadwiga Konieczna is granted with a PhD fellowship entitled "beca para la formación de personal investigador, en el marco de un programa operativo cofinanciado por el Fondo Social Europeo".

Conflict of interest: None

Abstract

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

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

Introduction

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

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

The aim of the current study was to identify early potentially predictive biomarkers of metabolic health by transcriptome profiling of PBMCs. As a model, we used the offspring of 20% maternal calorie-restricted dams during lactation, which are less prone to obesity development compared to *ad libitum* fed controls. We also ascertained i) to what extent the potential markers of optimized health identified at early ages continued to serve as potential markers in adulthood and ii) whether the changes occurring in blood cells reflected the metabolic environment in key tissues. We focused on selected genes of lipid metabolism and assessed these in relevant metabolic tissues, liver and white adipose tissue (WAT), at different ages.

Methods and Materials

Animals and experimental design

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

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

At weaning, a set of animals made up of 24 pups from control (12 males and 12 females) and 24 from CRL (12 males and 12 females) group were sacrificed by decapitation under *ad libitum* feeding conditions. One half of pups (n=6/group) were used to obtain different WAT depots (inguinal and retroperitoneal; iWAT and rWAT, respectively) and liver (which were rapidly removed, frozen in liquid nitrogen and stored at -70°C until RNA analysis), as well as trunk blood samples (for peripheral blood mononuclear cells (PBMCs) isolation, as described in the next section). From the other half of pups (n=6/group), blood samples were collected in heparinized containers and plasma was obtained by centrifugation at 700 g for 10 min.

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

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

Blood samples collected at the ages of 21 days (under *ad libitum* feeding conditions) and 6 months (under *ad libitum* and 12 h fasting conditions) were used for analysis of circulating parameters. Blood glucose concentration was measured by Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Peripheral hormones were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits: insulin concentration was determined using a rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden) and leptin with Quantikine™ Mouse Leptin Immunoassay (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Plasma non-esterified fatty acid (NEFA) and triglyceride levels (TG) were determined with a commercial enzymatic colorimetric kits (Wako Chemicals GmbH, Neuss, Germany and Triglyceride (INT) 20, Sigma Diagnostics, St Louis, MO, USA, respectively), following standard procedures. The homeostatic model assessment for insulin resistance (HOMA-IR) was used to assess insulin resistance. It is calculated from fasting insulin and glucose concentration using the formula of Matthews et al. (Matthews et al. 1985): $HOMA-IR = \text{fasting glucose (mmol/liter)} \times \text{fasting insulin (mU/liter)} / 22.5$.

PBMC isolation

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

Total RNA isolation

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

Microarray processing

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

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

Microarray data analysis

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

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

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

To validate microarray data, mRNA expression levels of apolipoprotein B48 receptor (*Apob48r*), carnitine palmitoyltransferase 1 alpha (*Cpt1a*), fatty acid synthase (*Fasn*), hormone-sensitive lipase (*Lipe*), low density lipoprotein receptor-related protein 1 (*Lrp1*), phosphate cytidylyltransferase 2, ethanolamine (*Pcyt2*), retinoid X receptor beta (*Rxb*), sortilin-related receptor, LDLR class A repeats-containing (*Sor11*) and steroidogenic acute regulatory protein (*Star*) were measured by RT-qPCR in PBMC RNA samples of control and CRL animals. Additionally, RT-qPCR was performed to analyze mRNA expression of aforementioned genes in PBMCs at the age of 6 months, and in iWAT, rWAT and liver, at the age of 21 days and 6 months. Regarding *Star*, its mRNA levels were only analyzed in liver, as its expression has not been described in WAT. We did not analyze *Apob48r* or *Sor11* expression either in liver nor in WAT, as both genes are expressed mainly in blood and neural cells.

For RT-qPCR analysis, 0.05 µg of PBMC total RNA was used for reverse transcription by using iScript™ cDNA synthesis kit (Bio-Rad Laboratories, S.A., Madrid, Spain) according to the manufacturer's protocol. For iWAT, rWAT and liver, 0.25 µg of total RNA (in a final volume of 5 µl) was firstly denatured at 65 °C for 10 min, and then reverse transcribed to cDNA with MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20 °C for 15 min, at 42 °C for 30 min, with a final step of 5 min at 95 °C in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain).

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

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

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon (bp)
<i>β-actin</i>	TACAGCTTCACCACCACAGC	TCTCCAGGGAGGAAGAGGAT	120
<i>Apob48r</i>	GGGCTACATCAGGCTTTGAG	TTCTCCCCTACAACCTTCC	150
<i>Cpt1a</i>	GCTCGCACATTACAAGGACAT	TGGACACCACATAGAGGCAG	250
<i>Fasn</i>	CGGCGAGTCTATGCCACTAT	ACACAGGGACCGAGTAAT	222
<i>Gdi-1</i>	CCGCACAAGGCAAATACATC	GACTCTCTGAACCGTCATCAA	210
<i>Lipe</i>	TCACGCTACATAAAGGCTGCT	CCACCCGTAAAGAGGGAACT	169
<i>Lrp1</i>	GAGCAGGTTGTCAGTCAGCA	TAGGGTTTCCGATTTCACA	187
<i>Pcyt2</i>	CCGACAGGGATGGGTCTG	TGGCTTCCTTCTTCTGATTCC	156
<i>Rxb</i>	CCCTCCCAGTCATCAGTTC	GGTGGCTTCACATCTTCAGG	152
<i>Sor11</i>	CACCGTCTCATTGTCAGCAC	ATCTCGTAGCCCTGGTTTC	123
<i>Star</i>	GGGTGGATGGGTCAGGTC	CTGCTGGCTTTCCTTCTTCC	168
<i>Tbp</i>	ACCCTTACCAATGACTCCTATG	ATGATGACTGCAGCAAATCGC	190

Abbreviations: apolipoprotein B48 receptor (*Apob48r*); carnitine palmitoyltransferase 1alpha (*Cpt1a*); fatty acid synthase (*Fasn*); GDP dissociation inhibitor 1 (*Gdi-1*); hormone-sensitive lipase (*Lipe*); low density lipoprotein receptor-related protein 1 (*Lrp1*); phosphate cytidylyltransferase 2, ethanolamine (*Pcyt2*); retinoid X receptor beta (*Rxb*); sortilin-related receptor, LDLR class A repeats-containing (*Sor11*); steroidogenic acute regulatory protein (*Star*) and TATA-box binding protein (*Tbp*).

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

Statistical analysis

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

Results

Phenotypic characteristics and blood parameters throughout life

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

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

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

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

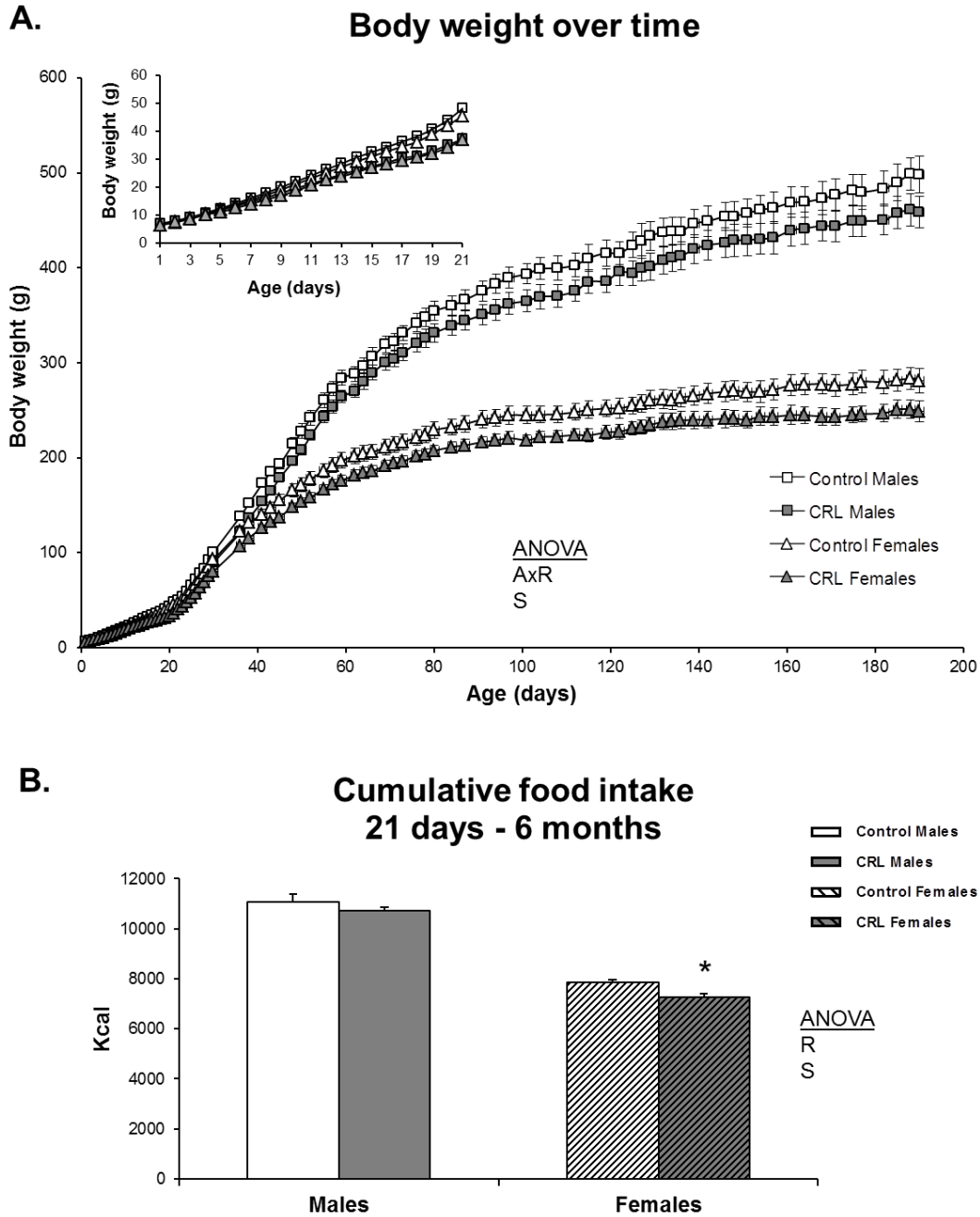


Figure 1A. Body weight with time of male and female offspring of control and calorie-restricted dams during lactation (CRL) from day 1 until day 190 of age. The inset details body weight throughout the lactating period (from day 1 until day 21 of age). Data are mean \pm S.E.M. ($n=12-16$ animals/group).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

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

			Males		Females		ANOVA
			Control	CRL	Control	CRL	
A) Morphological traits							
21 days	Body weight (g)		48.1 ± 2.1	37.1 ± 0.8*	45.4 ± 1.1	36.9 ± 0.5*	R
	Body length (cm)		11.5 ± 0.2	10.8 ± 0.2*	11.0 ± 0.2	10.4 ± 0.1*	R, S
	Body fat (%)		10.0 ± 0.4	8.68 ± 0.26*	10.5 ± 0.3	8.87 ± 0.19*	R
	iWAT (mg)		294 ± 35	187 ± 19*	307 ± 33	186 ± 26*	R
	rWAT (mg)		69.6 ± 9.9	40.1 ± 4.9*	50.5 ± 3.7	24.9 ± 3.2*	R
	Liver (g)		1.68 ± 0.05	1.25 ± 0.08*	1.76 ± 0.06	1.29 ± 0.03*	R
6 months	Body weight (g)		498 ± 19	459 ± 17	281 ± 12	249 ± 11	R, S
	Body length (cm)		24.2 ± 0.5	24.0 ± 0.3	20.8 ± 0.3	20.3 ± 0.2	S
	Body fat (%)		20.7 ± 2.2	22.1 ± 1.6	22.1 ± 2.3	14.5 ± 1.2*	RxS
	iWAT (g)		9.95 ± 1.20	10.7 ± 0.7	3.93 ± 0.37	2.14 ± 0.45*	RxS
	rWAT (g)		12.8 ± 1.7	12.4 ± 1.0	4.53 ± 0.67	2.16 ± 0.23*	RxS
	Liver (g)		16.0 ± 0.9	13.4 ± 0.6*	8.13 ± 0.47	7.35 ± 0.28	S, R
B) Circulating parameters							
21 days	Glucose (mg/dL)	Fed	137 ± 7	120 ± 3*	141 ± 5	116 ± 4*	R
	Insulin (ng/L)	Fed	0.111 ± 0.042	0.044 ± 0.010	0.105 ± 0.020	0.037 ± 0.004*	R
	Leptin (ng/L)	Fed	0.941 ± 0.154	0.408 ± 0.045*	1.05 ± 0.11	0.585 ± 0.162*	R
	NEFA (nM)	Fed	1.46 ± 0.13	1.50 ± 0.14	1.37 ± 0.19	1.59 ± 0.13	
	TG (mg/mL)	Fed	1.02 ± 0.14	0.893 ± 0.130	0.938 ± 0.094	0.851 ± 0.042	
6 months	Glucose (mg/dL)	Fed	108 ± 3	107 ± 6	105 ± 4	102 ± 4	
		Fasting	98 ± 8	95 ± 3#	102 ± 3	90 ± 1*#	R (p=0.059)
	Insulin (ng/L)	Fed	2.77 ± 0.94	1.44 ± 0.26	0.835 ± 0.102	0.459 ± 0.050*	R (p=0.069), S
		Fasting	0.920 ± 0.379#	1.06 ± 0.24#	0.558 ± 0.121#	0.287 ± 0.04#	
	HOMA-IR	Fasting	5.37 ± 2.38	5.77 ± 1.01	3.33 ± 0.75	1.67 ± 0.32	S
	Leptin (ng/L)	Fed	15.8 ± 2.2	13.4 ± 1.1	6.07 ± 0.94	1.74 ± 0.19*	RxS
	NEFA (nM)	Fed	0.851 ± 0.152	0.910 ± 0.056	1.33 ± 0.24	0.866 ± 0.086	
	TG (mg/mL)	Fed	1.61 ± 0.08	1.40 ± 0.19	1.02 ± 0.12	0.920 ± 0.073	S

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

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

At the age of 6 months, a tendency to lower glucose and insulin levels (under fasting and fed conditions, respectively) was found in CRL animals *versus* controls (p=0.059 and p=0.069,

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

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

In our microarray analysis, 45,018 probes were tested. Of them, those having an expression value of twice above the background (22,920) were further taken into account and normalized. In total 310 probes were found to be significantly different between control and CRL animals ($p \leq 0.01$; two-way ANOVA). Removal of duplicates resulted in 278 unique genes. Using available databases (Genecards, KEGG, NCBI, Reactome, UniProt, USCNC, WikiPathways), these genes were classified into several biological processes according to their function. 111 genes were unknown, thus were not included in any of the processes. From the remaining 167 known genes, 113 exhibited down-regulation and 54 up-regulation. As shown in Figure 2, the processes with the highest number of genes differentially expressed were related to immune system, signaling, cell turnover, transcription machinery and transport (28, 24, 19, 16 and 12 genes, respectively). Other processes with a notable number of genes were related to metabolism of proteins, carbohydrates and lipids (11, 11 and 9, respectively). The remaining genes were related with the nervous system, redox metabolism, cytoskeleton, neural signaling, blood, cell communication, central metabolism, sensory perception, and food intake control (9 or less genes involved in each of the processes).

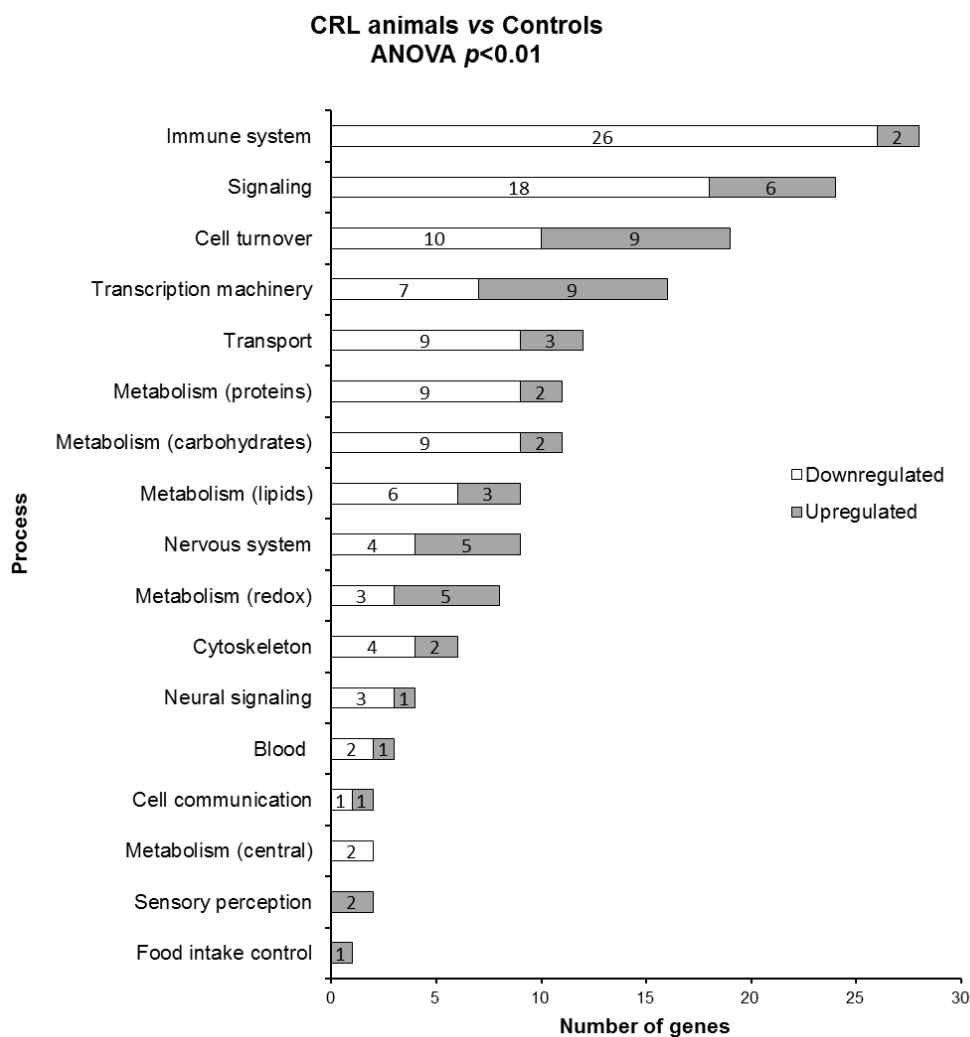


Figure 2. Classification into biological processes of the genes differentially expressed in PBMCs samples of male and female offspring of control and calorie-restricted dams during lactation (CRL) at the age of 21 days. Statistical analysis was performed by considering males and females as a whole ($p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

Subsequently, genes involved in lipid metabolism were analyzed in detail (Table 3). Of the 9 genes involved in this process (*Apob48r*, *Cpt1a*, *Fasn*, *Lipe*, *Lrp1*, *Pcyt2*, *Rxb*, *Sorl1*, *Star*), 6 showed down-regulation and 3 up-regulation. Down-regulated genes were involved in lipogenesis/lipolysis, glycerophospholipid biosynthesis and low density lipoprotein uptake. Up-regulated genes were involved in β -oxidation, lipolysis and cholesterol transport.

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

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

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

Confirmation of microarray results by RT-qPCR

To confirm gene array findings and to test whether the changes were consistent, RT-qPCR analysis of genes involved in the process of lipid metabolism was performed on the same RNA samples of 21-day-old male and female control and CRL animals. Genes chosen for confirmation were: *Fasn* (lipogenesis), *Rxb* (lipogenesis/lipolysis), *Cpt1a* (β -oxidation), and *Lipe* (lipolysis); *Apob48r*, *Lrp1*, and *Sor11* (all involved in lipoprotein uptake); *Pcyt2* (glycerophospholipid biosynthesis); *Star* (cholesterol transport and uptake).

RT-qPCR analysis confirmed most of the microarray data (Table 3), as differences in the expression levels of *Cpt1a*, *Lipe*, *Star* and *Fasn*, *Lrp1* and *Rxb* between control and CRL animals reached statistical significance ($p < 0.05$; two-way ANOVA) and followed the same pattern of up- and down-regulation as observed in the microarray analysis. Moreover, fold changes of those genes were similar using both techniques. Although RT-qPCR analysis of *Pcyt2* did not reveal significant differences between control and CRL animals, single comparison between groups revealed that CRL females exhibited a trend to lower *Pcyt2* mRNA levels relative to their controls ($p = 0.068$; Student's *t* test). Differences for *Apob48* and *Sor11* identified using the microarrays could not be confirmed by RT-qPCR analysis.

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

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

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

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

To evaluate how closely the changes in the lipid metabolism-related gene expression levels in PBMCs indicate those occurring in liver, iWAT and rWAT, correlation tests on the corresponding genes were performed (correlation values are indicated in Figure 3). Notably, *Cpt1* mRNA levels in PBMCs were positively correlated with their expression levels in iWAT, rWAT and liver; and also *Fasn* mRNA levels in PBMCs correlated positively with their

expression levels in iWAT and rWAT. *Star* mRNA levels in PBMCs were slightly correlated with *Star* mRNA levels in liver. On the other hand, no associations were found in relative mRNA expression responses of the other genes studied in PBMCs (*Lipe*, *Lrp1*, *Pcyt2* and *Rxb*) with those in WAT or liver (data not shown).

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

We also examined how the mRNA pattern of PBMC lipid metabolism-related genes, which were significantly altered at the age of 21 days in CRL rats, behaved in adult animals (Table 4). Our results show that overexpressed levels of *Star* in young CRL males and females were maintained in adulthood, although without reaching statistical significance ($p < 0.077$; two-way ANOVA). Changes found for *Fasn* expression levels in young animals were only preserved in adulthood for females ($p < 0.05$; Student's *t* test; interactive effect between moderate maternal calorie-restriction during lactation and sex, $p < 0.05$; two-way ANOVA). No changes in mRNA levels of *Apob48r*, *Cpt1a*, *Lipe*, *Lrp1*, *Pcyt2* and *Sor11* were observed between control and CRL animals, and, contrary to what was found in young animals, *Cpt1a* mRNA levels were down-regulated in CRL females relative to their controls ($p < 0.05$; Student's *t* test).

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

	Males		Females		ANOVA
	Control	CRL	Control	CRL	
<i>Apob48r</i>	100 ± 12	101 ± 12	100 ± 23	110 ± 20	
<i>Cpt1a</i>	100 ± 5	102 ± 8	114 ± 12	84.4 ± 3.6 *	
<i>Fasn</i>	100 ± 8	109 ± 8	126 ± 4	99.7 ± 4.8 *	RxS
<i>Lipe</i>	100 ± 13	107 ± 12	91.9 ± 5.2	92.4 ± 12.4	
<i>Lrp1</i>	100 ± 25	139 ± 39	73.8 ± 13.5	110 ± 13.2	
<i>Pcyt2</i>	100 ± 19	121 ± 28	84.1 ± 8.8	109 ± 22	
<i>Rxb</i>	100 ± 6	115 ± 6	125 ± 10	103 ± 4	RxS
<i>Sor11</i>	100 ± 21	96.5 ± 16.1	75.7 ± 2.5	103 ± 18	
<i>Star</i>	100 ± 15	137 ± 22	120 ± 10	167 ± 33	R ($p = 0.077$)

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

21 days old

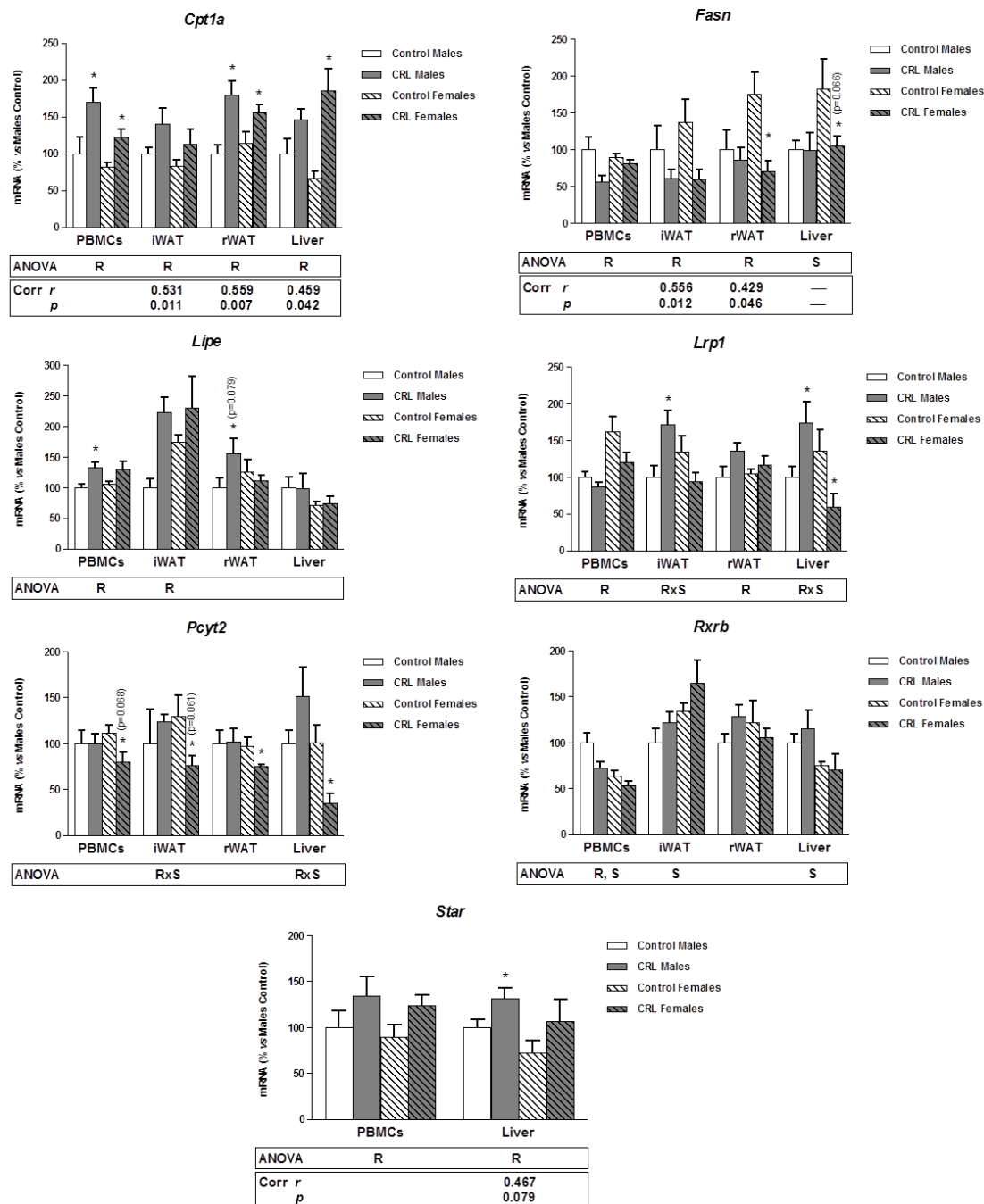


Figure 3. Comparison of mRNA expression levels of lipid metabolism-related genes in PBMCs, with the expression determined in inguinal and retroperitoneal WAT and liver of male and female offspring of control and calorie-restricted dams during lactation (CRL) at the age of 21 days. mRNA levels were measured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

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

We next determined if the genes showing differential expression between control and CRL adult animals in PBMCs, *Fasn*, *Cpt1a* and *Star*, also showed this in both fat depots (iWAT and rWAT) and in liver at an older age. We also included *Rxrb*, because its expression levels in control and CRL animals showed different patterns dependent on the sex of animals (Figure 4). Changes in *Cpt1a* mRNA levels observed in PBMCs of adult rats reflected those found in rWAT and liver (interactive effect between sex and calorie-restriction, $p < 0.05$; two-way ANOVA), although in rWAT changes did not reach statistical significance.

6 months old

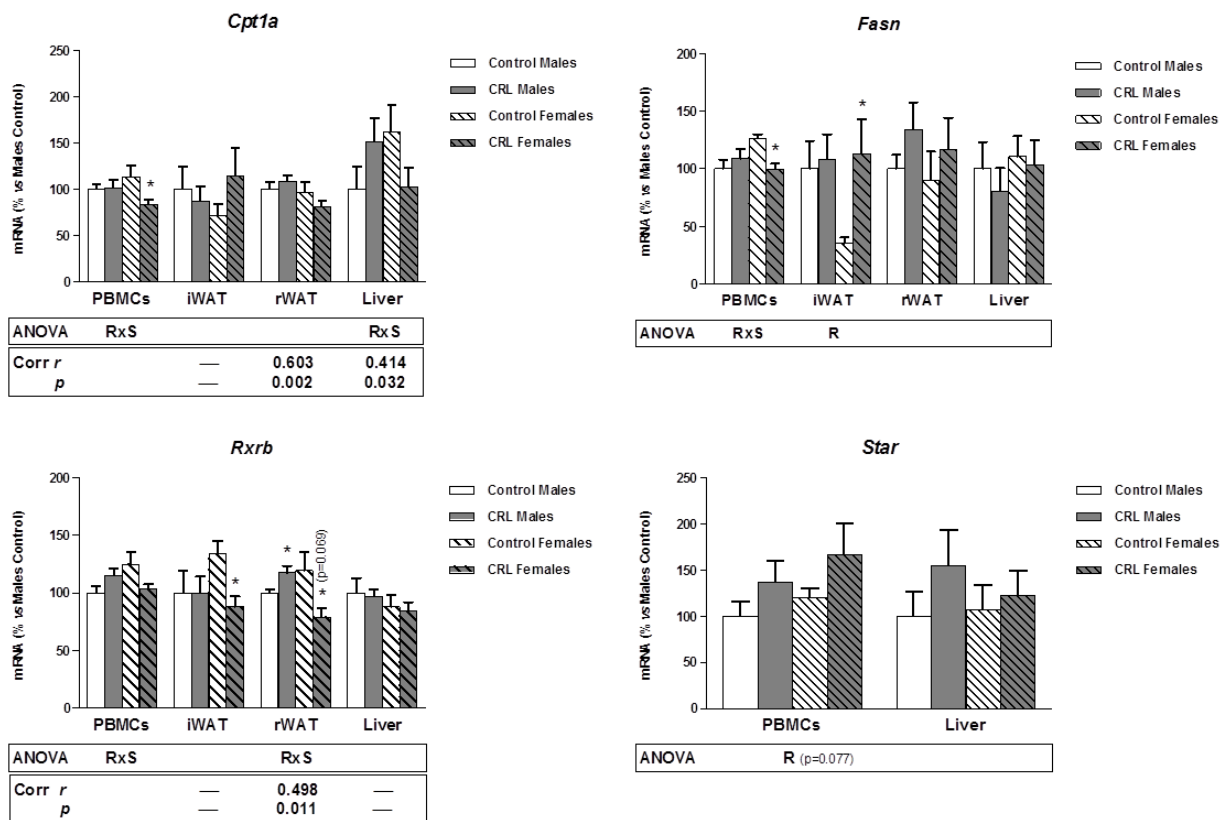


Figure 4. Comparison of mRNA expression levels of some lipid metabolism-related genes in PBMCs with the expression determined in inguinal and retroperitoneal WAT and liver of male and female offspring of control and at 6 months of age. Only those genes with significant differences between control and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

Concerning *Rxrb*, its mRNA levels in rWAT were differentially expressed in CRL animals depending on sex, the same as observed in PBMCs (interactive effect between sex and calorie-restriction, $p < 0.05$; two-way ANOVA). A similar trend was also found in iWAT for females ($p < 0.05$; Student's *t* test). *Star* expression profile observed in PBMCs was somewhat reflected in the liver, although without reaching statistical significance. Regarding *Fasn*, changes observed at mRNA levels of adult CRL animals with respect to controls in PBMCs were not reflected either in fat depots or in liver.

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

Discussion

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

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

Circulating hormones, such as insulin and leptin, could also contribute to characterize the metabolic health of these animals in relation to a better ability to maintain energy homeostasis throughout life. Leptin and insulin are hormones related to central control of feeding behavior and energy expenditure (Schwartz et al. 2000). High leptin levels are associated with insulin resistance and metabolic syndrome (Esteghamati et al. 2009), whereas lower leptin levels have

been associated with improvement of insulin sensitivity (Sanchez et al. 2008). At weaning, CRL animals showed lower plasma levels of insulin and leptin. This healthier profile of circulating hormones was sustained in adulthood, particularly in females.

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

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

The hormone-sensitive lipase (LIPE, better known as HSL), encoded by the *Lipe* gene, is an enzyme that hydrolyzes acylglycerols, as well as several other lipids stored in adipose tissue (Yeaman 2004). Although transcription of *Lipe* was initially described as specific for adipocytes, subsequent studies have shown that it is expressed and has a role in lipid metabolism in multiple tissues, including liver and macrophages (Yeaman 2004). Its action has also been suggested to be linked with insulin secretion and insulin action (Kraemer and Shen 2002). Expression of this gene has also been described in PBMCs, where its level was found to be reduced in “at risk” obese subjects (Telle-Hansen et al. 2013). Interestingly, in the present study we show that young CRL animals exhibited higher *Lipe* mRNA levels in PBMCs and similarly in iWAT. Therefore, higher mRNA levels of this gene might be proposed as an early marker for a better capacity to regulate lipid metabolism in adulthood, possibly in association with improved insulin sensitivity. However, early changes were not persistent into adulthood, suggesting their potential usefulness as a biomarker only at early ages.

Cpt1a codes for carnitine palmitoyltransferase 1alpha (liver form). It mediates the transport of long-chain fatty-acids across the mitochondrial inner membrane and is rate limiting for their beta-oxidation. High expression levels of *Cpt1a* in PBMCs have been described in diet-induced obese rats (Caimari et al. 2010b). Similarly, *Cpt1a* mRNA levels in whole blood cells were higher in those overweight male children that seemed to be protected against the increase in plasma triglyceride levels associated with body fat accumulation (Sanchez et al. 2012). Conversely, lower expression of this gene in overweight male children was associated with higher HOMA index (Sanchez et al. 2012). In the present study, young CRL animals showed

higher mRNA levels of *Cpt1a* in PBMCs than controls. Moreover, this expression pattern was correlated with that occurring in liver and WAT (both inguinal and retroperitoneal depots). However, differences found at early ages were not sustained in adulthood, neither in PBMCs nor in the tissues that were investigated, although the expression profile of this gene in PBMCs of adult animals was also related to the expression in rWAT and liver. Altogether, these results suggest positive metabolic effects related with high expression levels of *Cpt1a* in PBMCs at early ages in relation to increased oxidative capacity.

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

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

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

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

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

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

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7

*Moderate maternal calorie restriction
in lactating rats affects markers of
protein damage by glycation and
oxidation in breast milk*

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Thornalley PJ.

(to be submitted)

Title page

Title: Moderate maternal calorie restriction in lactating rats affects markers of protein damage by glycation and oxidation in breast milk

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Statement of financial support: We gratefully acknowledge the Spanish Government (grant AGL2012-33692) and the European Union (BIOCLAIMS FP7-244995). The Laboratory of Molecular Biology, Nutrition, and Biotechnology (Nutrigenomics) is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, EU Contract: n° FP6-506360). The CIBER de Fisiopatología de la obesidad y nutrición is an initiative of the ISCIII.

Conflict of interest: None

Abstract

Introduction: Advanced glycation end products (AGEs) and other protein damage biomarkers are contributors to oxidative stress and inflammatory response. Calorie restriction slows the age-related processes by reduction of oxidant stress and, in lactating rats, this condition has been shown to protect the offspring against the development of obesity and metabolic-related alterations.

Aim: To assess whether the beneficial effects of moderate maternal calorie restriction during lactation in rats on their offspring might be related with changes in protein damage markers in milk.

Methods: A quantitative screening of glycation, oxidation and nitration free adducts and of individual free amino acids was performed in maternal plasma (day 21 of lactation) and milk samples (days 5, 10 and 15 of lactation) from control and 20% calorie restricted (CRL) lactating dams by using chromatographic techniques with mass spectrometric detection and quantification using stable isotope substituted standards.

Results: In plasma, CRL dams showed lower levels of most amino acids and of the free adducts MG-H1, 3DG-H, FL, MOLD and AASA than controls. In milk, differences were depending on the stage of lactation. Compared to controls, milk from CRL dams contained higher CEL, GH1, CMA, CML, 3DG-H, Pyrraline, Dityrosine and AASA levels at day 10 of lactation, but it contained lower levels of free amino acids and of MG-H1, Pyrraline and NFK at day 15.

Conclusion: The presence of diminished glycation and oxidation free adducts levels in milk from CRL dams during the second part of lactation might contribute to, and could be a marker of factors contributing to the health benefits of this condition in the offspring.

Keywords: damage protein, LC-MS/MS, breast milk, calorie restriction

Introduction

Protein damage by glycation, oxidation or nitration processes, and hence, formation of advanced glycation end products (AGEs), protein oxidation or nitration adducts, respectively, play an important role in the pathogenesis of aging-related chronic diseases, such as obesity, diabetes and cardiovascular and renal disorders (Singh, et al. 2014). Advanced glycation is one of the major pathways involved in the development and progression of different diabetic complications. Moreover, several studies show an association between high plasma levels of AGEs and a wide range of harmful health effects, such as cardiovascular disease, Alzheimer's disease, cancer, uraemia, type I and II diabetes, aging and pro-oxidative and pro-inflammatory events (Klenovics, et al. 2013; Thornalley, et al. 2003).

AGEs are formed during a complex series of parallel, sequential and non-enzymatic reactions between the free amino groups of proteins and carbonyl groups of reducing sugars or other carbonyl compounds; these reactions are known as the Maillard reactions or browning reactions (Thornalley 2008). At early stages of glycation reactions, glucose reacts with lysine residue side-chain amino groups to form early glycation adducts, a Schiff base adduct that slowly rearranges to N ϵ -fructoselysine (FL) (Rabbani and Thornalley 2009). In later stage reactions, FL adducts degrade slowly to form irreversibly AGEs through oxidation, dehydration and cyclization reactions (Singh, et al. 2014). AGEs are yellow-brown insoluble adducts that accumulate on long-lived proteins interfering with their normal functions by disrupting molecular conformation, altering enzymatic activity and interfering with receptor recognition (Singh, et al. 2014). AGEs-modified proteins undergo accelerated degradation by the proteasome to form free-AGEs such as 3-deoxyglucosone (3-DG), N δ -(5-hydro-4-imidazol-2-yl)ornithine (G-H1), N δ -(5-hydro-5-methyl-4-imidazol-2-yl)ornithine (MG-H1), N ϵ -carboxymethyl-lysine (CML), N ϵ -carboxyethyl-lysine (CEL) and N ω -carboxymethylarginine (CMA) (Rabbani and Thornalley 2009; Thornalley 2008).

Concerning protein oxidation adducts, they are formed mainly by exposure of amino acids residues in proteins to hydrogen peroxide, hypochlorite and peroxyxynitrite, and methionine sulfoxide residues (MetSO), dityrosine and N-formylkynurenine (NFK) are important markers of protein oxidation (Rabbani and Thornalley 2009). Protein nitration adducts, such as 3-Nitrotyrosine (3-NT) are formed by exposure of proteins to peroxyxynitrite and nitryl chloride (Droge 2002).

AGEs are formed endogenously in the body and their formation progressively increases with normal ageing and also in association with hyperglycemia, oxidative or carbonyl stress and decreased renal function (Sebekova and Somoza 2007). Furthermore, humans are also exposed to exogenous AGEs, which are ingested with food. Industrial processing of some saccharide-rich foods requires heat-treatment; this results in the formation of substantial amounts of AGEs, especially CML, which is considered a stable compound and a good indicator of AGEs in food. It has been recently demonstrated that AGEs from saccharide-rich foods are at least partially absorbed from the gastrointestinal tract contributing to the circulating pool of AGEs (Sebekova, et al. 2008; Sebekova and Somoza 2007). Human studies have recently revealed significant correlations between ingested AGEs, circulating AGEs and several markers of inflammation (Peppas, et al. 2004; Vlassara, et al. 2002). In experimental animals, high consumption of heat treated foods, which are a fuel of high levels of exogenous AGEs, has been shown to promote the manifestation of diabetes, and to substantially contribute to the acceleration of the development of diabetic complications (Sebekova and Somoza 2007) and to renal and vascular complications (Singh, et al. 2014). According to this, a long-term AGE restriction diet,

although isocaloric to the standard diet, has been described to prevent age-associated weight gain and insulin resistance in mice, suggesting that this dietary intervention may preserve mechanisms related to glucose and energy utilization, which become impaired with aging (Cai 2004).

Likewise, reduced calorie intake has been found to delay the age-related processes and reduce oxidant stress in mammals [reviewed by (Vlassara 2005)]. This has been associated, in part, to the reduced intake of dietary AGEs accompanied to the reduction in food consumption (Cai, et al. 2008). In our previous studies we have shown that moderate calorie restriction of rat dams during lactation also protects their offspring against diet-induced obesity and related metabolic alterations, particularly dyslipidemia, insulin resistance, and hyperleptinemia (Palou, et al. 2010a; Palou, et al. 2011; Torrens, et al. 2014). Therefore, we hypothesized that this condition during lactation may also result in a reduction in AGE consumption and hence a reduction in their circulating levels. Moreover, it might be speculated that the content of AGEs and other adducts in milk might also be affected, which may potentially contribute to the aforementioned beneficial effects in their offspring.

Therefore, considering that i) AGE formation is a contributor of oxidative stress and inflammatory response, ii) calorie restriction slows the age-related processes by reduction of oxidant stress, and iii) moderate maternal calorie restriction in lactating dams confers some protection against obesity and metabolic-related alterations in the offspring, here we aimed to determine whether moderate (20%) maternal calorie restriction during lactation is able to reduce the amount of AGEs and other markers of protein damage by oxidation and nitration in maternal plasma, as well as in breast milk. To achieve this, we performed a quantitative screening of free amino acids and of a comprehensive range of free glycation, oxidation and nitration adducts in maternal plasma and breast milk samples from control and CRL dams by using chromatographic techniques with mass spectrometric detection and quantification using stable isotope substituted standards.

Materials and Methods

Animals and experimental design

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

The study was performed in female Wistar rats weighing between 225 and 260 g (Charles River Laboratories, Barcelona, Spain). Animals were housed under standard conditions, that is, controlled temperature (22°C), a 12 h light-dark cycle, and free access to tap water. Briefly, 22 virgin female rats were mated with male rats (Charles River Laboratories, Barcelona, Spain). After matching, each female was placed in an individual cage. At day 1 after delivery, pups were weighed and the size of all litters was adjusted to 10 neonates with similar body weight per mother (5 males and 5 females, when possible) to avoid differences in milk production due to a different number/weight of pups per dam to feed. Dams were assigned to either control (n=11 dams) or calorie restricted (n=11 dams) groups. Throughout the lactating period, starting on day 1 after delivery until weaning (day 21), control dams were fed *ad libitum* with standard chow diet (3 kcal/g, with 8% calories from fat; Panlab, Barcelona, Spain), while calorie restricted (CRL) dams underwent 20% restriction of calorie intake. Calorie restriction was

performed by offering each dam a daily amount of food corresponding to 80% of the calories they should eat according to their body weight. This amount was calculated by considering the calories consumed daily by their control animals under *ad libitum* feeding conditions.

Breast milk samples of each control and CRL dams were collected on days 5, 10 and 15 of lactation. Before milking, nursing rats were separated from their pups between 2-3 h to guarantee that mammary glands were full of milk. Milk collection was performed on isoflurane-anesthetized rats. To induce milk letdown, approximately 5 min after anesthetizing the animals, an intraperitoneal injection of 0.4 IU/Kg of oxytocin was applied (Facilpart. Oxytocin 10 IU/mL; Laboratories Syva. León, Spain). Milk samples were collected in sterilized tubes and stored at -80°C until stable isotopic dilution analysis liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed.

At weaning (day 21 of lactation), control and CRL dams were killed under *ad libitum* feeding conditions by decapitation during the first 2 h at the beginning of the light cycle. Trunk blood samples were collected in heparinized containers. Plasma was obtained by centrifugation of heparinized blood at 1000 x g for 10 min and stored at -80°C until LC-MS/MS analysis.

Stable isotopic dilution analysis liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Ultrafiltrate of maternal plasma (from day 21 of lactation) and ultrafiltrate of breast milk samples (from days 5, 10 and 15 of lactation) of control and CRL dams were analyzed by LC-MS/MS to perform a quantitative screening of all the amino acids and a comprehensive range of protein damage biomarkers. The following free glycation adducts were determined: MG-H1, 3DG-H, Glucosepane, G-H1, CMA, CML, CEL, Fructosyl-lysine (FL), bis(lysyl) crosslink derived from methylglyoxal (MOLD) and pyrraline. Concerning free oxidation adducts: amino adipic semialdehyde (AASA), glutamic semialdehyde (GSA), dityrosine, MetSO and NFK were measured. The free nitration adduct 3-NT was also measured (Table 1).

Ultrafiltrates of milk and plasma samples were prepared by either microspin ultrafiltration of 50 µl of milk or 60 µl of plasma using a 3 KDa or 10 KDa cut-off filter respectively, and then centrifuged at 14000 x g (milk samples for 2 h and plasma samples for 30 min) at 4°C. Ultrafiltrates were stored at -80°C until preparation of vials for ultrahigh performance liquid chromatography (UPLC).

UPLC vials were prepared by adding 5 µl of milk ultrafiltrate or 5 µl of plasma ultrafiltrate with 20 µl of 0.1% Trifluoroacetic acid (TFA) in water and 25 µl of a cocktail of stable isotope substituted internal standards.

Chromatography was performed on two HypercarbTM graphic 5 µm particle size columns (2.1 x 50 mm and 2.1 x 250 mm, respectively. Thermo Hypersil Ltd). The chromatographic procedure was customized to complete data collection of all analytes of each sample in a 35-minute run. The mobile phase was solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 50% acetonitrile (ACN)). The two columns were initially used in series with a flow rate of 0.2 mL/min and later (at 20 min), the second column was then switched out of the flow to facilitate elution of strongly hydrophobic analytes. The post-run method, for washing, used solvent A1 (0.1% TFA in water) and solvent B2 (0.1% TFA in 50% tetrahydrofuran (THF)) for 30 minutes. Optimised custom gradients were used for analysis and post-run column wash/re-equilibration runs. Flow from the column in the interval 4-35 min was directed to the mass spectrometry (MS/MS) detector. Each analyte was detected by electrospray positive ionization

mass spectrometric multiple reaction monitoring (MRM). The ionization source of temperature was 150°C and the desolvation gas temperature 500°C. The cone gas and desolvation gas flow were 150 L/h and 1000 L/h, respectively. Each analyte was detected by selective ion monitoring of its molecular ion in the first mass analyser after fragmentation with argon atoms in the collision cell. Therefore, each analyte was detected by characteristic chromatographic retention time, molecular ion mass/charge ratio m/z and fragmentation ion m/z . Programmed molecular ion and fragment ion masses and collision energies were optimized to ± 0.1 Da and ± 1 eV, respectively, for MRM detection of analytes. Detector response was normalized to the added stable isotopic standard response which has the same retention time as the normal analyte and a different molecular ion or molecular ion and fragment ion m/z value. Data were collected sequentially and rapidly for many mass transitions (up to 32) such that multiple mass chromatograms may be recorded for each analyte and isotopic standard. Analyte content was determined from the analyte/internal standard peak response peak area ratio by reference to calibration curves recorded under the same conditions with the same content of internal standard as the samples and known amounts of normal reference standard analyte.

Statistical analysis

Data are mean \pm standard error of the mean (S.E.M). Kolmogorov–Smirnov test was performed to determine whether the data set was normally distributed. Single comparisons between control and CRL dams were assessed by Student's t test (when samples were normally distributed) or by Mann-Whitney U test (when samples were no normally distributed). Finally, Bonferroni correction was applied to prevent data from incorrectly appearing to be statistically significant by lowering the alpha value. Multiple comparisons were assessed by repeated measured analysis of variance (ANOVA). Differences between the three days of milk collection during lactation, in both control and CRL dams, were assessed by Bonferroni post hoc test. The analyses were performed with SPSS for Windows (SPSS version 19.0, Chicago, IL). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. When Bonferroni correction was applied, alpha value was divided by the number of tests ($p\text{-value} = \alpha/n$).

Table 1. Mass spectrometric multiple reaction monitoring detection of damage protein biomarkers and of their respective internal standards

Analyte group	Analyte	Parent ion (Da)	Fragment ion (Da)	CE (eV)	Rt	Internal standard	Parent ion (Da)	Fragment ion (Da)	CE (eV)	Rt
Amino acids	Leucine	132.30	86.20	10	28.3	[² H ₃]Leucine	135.30	89.20	10	28.3
	Isoleucine	132.28	86.16	10	31.4	[¹³ C ₆]Isoleucine	138.28	91.20	10	31.4
	Valine	118.05	72.08	10	7.8	[² H ₈]Valine	126.05	80.08	10	7.8
	Arginine	176.20	71.06	18	32.2	[¹⁵ N ₂]Arginine	178.20	71.06	18	32.2
	Lysine	147.13	84.11	14	5.5	[¹³ C ₆]Lysine	153.10	89.11	14	5.5
	Histidine	156.10	110.08	14	32.1	[¹³ C ₆]Histidine	162.10	115.08	14	32.1
	Aspartic acid	134.04	88.03	10	7.0	[¹³ C ₂ - ¹⁵ N]Aspartic acid	137.04	90.03	10	7.0
	Glutamic acid	148.13	102.09	10	28.3	[¹³ C ₅]Glutamic acid	153.13	106.09	10	28.3
	Asparagine	133.20	87.18	8	6.6	[¹³ C ₄ H ₂ O]Asparagine	137.20	90.18	8	6.6
	Glutamine	147.24	84.12	16	8.8	[¹³ C ₅]Glutamine	152.20	88.10	16	8.8
	Serine	105.99	42.04	18	5.1	[¹³ C ₃]Serine	108.99	44.04	18	5.1
	Threonine	120.07	74.02	10	6.0	[¹³ C ₄ - ¹⁵ N]Threonine	125.07	78.02	10	6.0
	Tyrosine	182.90	137.00	12	18.4	[² H ₄]Tyrosine	186.90	141.00	12	18.4
	Tryptophan	206.07	159.80	15	23.7	[¹⁵ N ₂]Tryptophan	208.70	161.08	15	23.7
	Phenylalanine	167.10	121.15	12	17.1	[D ₅]Phenylalanine	172.10	126.15	12	17.1
	Cysteine	122.04	76.00	22	6.3	[¹³ C ₃ - ¹⁵ N]Cysteine	126.04	79.00	22	6.3
	Alanine	90.06	44.06	8	5.2	[D ₃]Alanine	93.06	47.06	8	5.2
	Proline	117.13	71.08	12	6.1	[¹³ C ₅]Proline	122.13	75.08	12	6.1
	Glycine	76.23	30.16	6	4.8	[¹³ C ₂ - ¹⁵ N]Glycine	79.23	32.16	6	4.8
Methionine	150.00	104.04	10	32.2	[² H ₃]Methionine	153.00	107.04	10	32.2	
Ornithine	133.02	70.08	14	5.0	[² H ₆]Ornithine	139.02	76.08	14	5.0	
Glycation adducts	MG-H1	229.16	114.10	16	11.9	[¹⁵ N ₂]MG-H1	231.16	116.10	16	11.9
	3DG-H	319.10	70.06	28	11.7	[¹⁵ N ₂]3DG-H	321.10	70.06	28	11.7
	Glucosepane	429.20	269.20	36	16.4	[¹³ C ₆]Glucosepane	435.20	275.10	36	16.4
	G-H1	215.17	100.05	12	12.7	[¹⁵ N ₂]G-H1	217.17	102.05	12	12.7
	CMA	233.10	70.13	28	12.3	[¹³ C ₂]CMA	235.10	70.13	28	12.3
	CML	205.12	84.12	16	30.0	[¹³ C ₆]CML	211.12	89.12	16	30.0
	FL	291.10	84.11	26	32.1	[² H ₄]FL	295.10	88.11	26	32.1
	MOLD	341.22	83.92	32	15.0	[² H ₈]MOLD	349.22	87.92	32	15.0
	Pyrraline	255.07	175.06	23	19.0	[² H ₄]Pyrraline	263.07	182.06	23	19.0
	CEL	219.17	130.04	12	32.2	[¹³ C ₃]CEL	225.17	136.04	12	32.2
Oxidation and nitration adducts	AASA	128.10	82.06	12	10.7	[D ₃]AASA	164.96	100.79	12	10.7
	GSA	114.02	68.08	15	32.2	[D ₃]AASA	164.96	100.79	12	10.7
	NFK	237.08	191.10	9	23.7	[¹⁵ N ₂]NFK	239.08	193.10	9	23.7
	Dityrosine	361.10	315.19	14	20.3	[² H ₆]Dityrosine	367.10	321.19	14	20.3
	MetSO	166.00	56.09	16	8.4	[² H ₃]MetSO	169.00	56.09	16	8.4
3-NT	227.12	181.07	12	23.4	[² H ₃]3-NT	230.12	184.07	12	23.4	

Molecular weight, chromatographic retention times and mass-spectrometric multiple reaction monitoring detection of free glycation, oxidation and nitration adducts and their respective internal standards. Abbreviations: CE, collision energy; Rt, retention time; MG-H1, Nδ-(5-hydro-5-methyl-4-imidazolone-2-yl)ornithine; 3DG-H, 3-deoxyglucosone; G-H1, Nδ-(5-hydro-4-imidazolone-2-yl)ornithine; CMA, Nω-carboxymethylarginine; CML, Nε-carboxymethyl-lysine; FL, Fructosyl-lysine; MOLD, bis(lysyl) crosslink derived from methylglyoxal; CEL, Nε-carboxyethyl-lysine; AASA, amino adipic semialdehyde; GSA, glutamic semialdehyde; NFK, N-formylkynurenine; MetSO, methionine sulfoxide; 3-NT, 3-Nitrotyrosine.

Results

Amino acid composition and free glycation, oxidation and nitration adducts in the ultrafiltrate of plasma samples from control and CRL dams at day 21 of lactation

Concentrations of the 21 amino acids analyzed in the ultrafiltrate of maternal plasma samples are shown in table 2. The concentration of total amino acids, as well as of individual amino acids, with the exception of aspartic acid, glutamic acid, glutamine, tryptophan, cysteine and glycine, in the plasma ultrafiltrate of CRL dams was significantly lower compared to that of control dams ($p < 0.0023$ by Student's *t* test and Mann-Whitney *U* test).

Table 3 shows the quantitative screening of free glycation, oxidation and nitration adducts in the ultrafiltrates of maternal plasma at day 21 of lactation. Notably, plasma of CRL dams showed significantly lower amounts of several free glycation adducts, MG-H1, 3DG-H, FL and pyrroline, as well as of the free oxidation adduct AASA, compared to plasma of control dams ($p < 0.005$, by Mann-Whitney *U* test). No significant differences were observed for the other free adducts analyzed.

Table 2. Amino acid composition in the ultrafiltrate of maternal plasma samples from day 21 of lactation

	Amino acid	Control (µM)	CRL (µM)
Branched-chain amino acids (BCAA)	Leucine	421 ± 15	261 ± 20 ###
	Isoleucine	429 ± 13	266 ± 24 ###
	Valine	648 ± 15	418 ± 30 ###
	Sum of BCAA	1498 ± 41	945 ± 73 ###
Basic amino acids	Arginine	457 ± 26	282 ± 30 #
	Lysine	2095 ± 87	1224 ± 84 ###
	Histidine	264 ± 7	216 ± 10 #
Acid amino acids	Aspartic acid	232 ± 13	197 ± 17
	Glutamic acid	625 ± 44	628 ± 44
Amidic amino acids	Asparagine	174 ± 7	119 ± 7 ###
	Glutamine	2258 ± 65	1975 ± 93
Hydroxyl amino acids	Serine	1339 ± 49	1012 ± 69 #
	Threonine	882 ± 54	418 ± 39 ###
Aromatic amino acids	Tyrosine	349 ± 16	199 ± 19 ###
	Tryptophan	1295 ± 51	1612 ± 176
	Phenylalanine	200 ± 5	153 ± 9 #
Other amino acids	Cysteine	31.4 ± 5.2	20.7 ± 1.9
	Alanine	1842 ± 82	1039 ± 105 ###
	Proline	3951 ± 125	1392 ± 145 ###
	Glycine	772 ± 65	716 ± 78
	Methionine	205 ± 7	110 ± 8 ###
	Ornithine	9.32 ± 0.76	5.60 ± 1.00 #
Sum	Total amino acids	18478 ± 278	12263 ± 685 ###

Data are mean ± S.E.M (n=11 animals per group) of control dams and moderate calorie restricted dams during lactation (CRL). Statistics: Kolmogorov-Smirnov test was used for testing the normality of the distribution of the samples. Differences between CRL and control were assessed by Mann-Whitney *U* test (for no normally distributed samples) or by Student's *t* test (for normally distributed samples) and Bonferroni correction for 21 analytes was applied: #, ## and ###, CR vs control when $p < 0.0023$, $p < 0.0005$ and $p < 0.00005$ respectively.

Table 3. Free glycation, oxidation and nitration adduct concentration in the ultrafiltrate of maternal plasma samples from day 21 of lactation

	Free adduct	Control (nM)	CRL (nM)
Glycation adducts	MG-H1	766 ± 44	396 ± 47 ***
	3DG-H	285 ± 20	195 ± 13 *
	Glucosepane	8.96 ± 1.34	10.0 ± 0.7
	G-H1	102 ± 9	89.8 ± 6.3
	CMA	95.3 ± 4.7	74.5 ± 5.5
	CML	182 ± 23	85.8 ± 8.6 **
	FL	1860 ± 137	993 ± 127 **
	MOLD	6.36 ± 0.97	5.13 ± 0.59 ***
	Pyrraline	1104 ± 70	329 ± 42
	CEL	1538 ± 105	2319 ± 387
Oxidation and nitration adducts	AASA	19.9 ± 1.1	11.9 ± 1.1 ***
	GSA	90.8 ± 3.5	115 ± 8
	NFK	9.43 ± 1.60	21.5 ± 4.3
	Dityrosine	12.6 ± 0.9	11.6 ± 0.7
	MetSO	2315 ± 146	1762 ± 131
	3-NT	3.06 ± 0.21	2.82 ± 0.15

Data are mean ± S.E.M (n=11 animals per group) of control dams and moderate calorie restricted dams during lactation (CRL). Statistics for glycation adducts: *, ** and ***, CRL respect to control when $p < 0.005$, $p < 0.001$ and $p < 0.0001$ respectively, by Mann-Whitney *U* test (followed by a Bonferroni correction for 10 analytes). Statistics for oxidation and nitration adducts: *, ** and ***, CRL respect to control when $p < 0.008$, $p < 0.002$ and $p < 0.0002$ respectively, by Mann-Whitney *U* test (followed by a Bonferroni correction for 6 analytes). Abbreviations: MG-H1, N δ -(5-hydro-5-methyl-4-imidazolone-2-yl)ornithine; 3DG-H, 3-deoxyglucosone; G-H1, N δ -(5-hydro-4-imidazolone-2-yl)ornithine; CMA, N ω -carboxymethylarginine; CML, N ϵ -carboxymethyl-lysine; FL, Fructosyl-lysine; MOLD, bis(lysyl) crosslink derived from methylglyoxal; CEL, N ϵ -carboxyethyl-lysine; AASA, amino adipic semialdehyde; GSA, glutamic semialdehyde; NFK, N-formylkynurenine; MetSO, methionine sulfoxide; 3-NT, 3-Nitrotyrosine.

Amino acid composition and free glycation, oxidation and nitration adducts in the ultrafiltrate of milk samples from control and CRL dams at days 5, 10 and 15 of lactation

Table 4 shows the concentration of the 21 amino acids analyzed in the ultrafiltrate of milk samples at different time points of lactation. For most of the amino acids, differences between control and CRL dams were dependent on the day of lactation; in fact a significant interaction was found between the effect of maternal calorie restriction during lactation and the time point of lactation for the sum of the concentrations of all amino acids and for most of the amino acids with the exception of Leucine, Lysine, Tyrosine and Ornithine, which showed a significant effect of the lactation time ($p < 0.05$, ANOVA repeated measures). Specifically, at days 5 and 10 of lactation, levels of methionine and arginine, respectively, were significantly higher in milk from CRL dams compared to milk from control animals ($p < 0.0023$, by Student's *t* test and $p < 0.00005$ by Mann-Whitney *U* test, respectively). In turn, at day 15 of lactation, Serine levels in milk from CRL dams were significantly lower compared to milk from control dams ($p < 0.00005$, by Student's *t* test). Notably, the concentration of most of the amino acids changed with the time of lactation in both control and CRL animals, with different patterns depending on the group of animals. Although the pattern was dependent on the specific amino acid, as a general trend, amino acid levels in milk increased from day 5 to 10 of lactation in both control and CRL animals. At day 15 of lactation, levels were maintained stable or continued to increase in control animals, but decreased to levels similar of those of day 5 of lactation in CRL dams. The sum of total amino acids also followed this trend, and milk of CRL dams at day 15 of lactation showed lower concentration of amino acids to that of control animals.

Table 4. Amino acid composition in the ultrafiltrate of breast milk samples from day 5, 10 and 15 of lactation

	Amino acid	Dam	Day 5 (µM)	Day 10 (µM)	Day 15 (µM)	ANOVA
Branched-chain amino acids (BCAA)	Leucine	Control	9.25 ± 1.07 <i>a</i>	17.7 ± 2.4 <i>a</i>	25.7 ± 4.1 <i>b</i>	<i>T</i>
		CRL	6.22 ± 0.64 <i>x</i>	19.1 ± 3.2 <i>y</i>	18.8 ± 3.8 <i>y</i>	
	Isoleucine	Control	11.4 ± 1.3 <i>a</i>	22.5 ± 2.7 <i>b</i>	26.1 ± 3.8 <i>b</i>	<i>TxR</i>
		CRL	10.1 ± 0.7 <i>x</i>	26.0 ± 2.0 <i>y</i>	17.9 ± 2.4 <i>z</i>	
	Valine	Control	17.4 ± 1.7 <i>a</i>	42.5 ± 5.4 <i>b</i>	56.6 ± 8.1 <i>c</i>	<i>TxR</i>
		CRL	16.6 ± 1.3 <i>x</i>	56.5 ± 7.4 <i>y</i>	34.0 ± 5.3 <i>z</i>	
Sum of BCAA		Control	38.1 ± 3.9 <i>a</i>	82.7 ± 10.3 <i>b</i>	108 ± 14 <i>b</i>	<i>TxR</i>
		CRL	32.9 ± 2.4 <i>x</i>	102 ± 13 <i>y</i>	71 ± 11 <i>z</i> #	
Basic amino acids	Arginine	Control	39.0 ± 3.3 <i>a</i>	59.7 ± 2.1 <i>b</i>	49.6 ± 5.2 <i>ab</i>	<i>TxR</i>
		CRL	48.5 ± 3.3 <i>x</i>	105 ± 7 <i>y</i> ###	41.5 ± 4.6 <i>x</i>	
	Lysine	Control	33.0 ± 4.3 <i>a</i>	70.2 ± 7.6 <i>b</i>	61.0 ± 8.7 <i>ab</i>	<i>T</i>
		CRL	37.9 ± 5.3 <i>xy</i>	91.5 ± 21.6 <i>x</i>	51.0 ± 12.7 <i>y</i>	
	Histidine	Control	10.9 ± 1.1 <i>a</i>	17.6 ± 1.2 <i>b</i>	24.1 ± 2.5 <i>c</i>	<i>TxR</i>
		CRL	11.1 ± 1.3 <i>x</i>	29.7 ± 6.1 <i>y</i>	24.1 ± 3.4 <i>y</i>	
Acid amino acids	Aspartic acid	Control	77.5 ± 3.9 <i>a</i>	183 ± 29 <i>b</i>	359 ± 61 <i>c</i>	<i>TxR</i>
		CRL	111 ± 8 <i>x</i>	262 ± 28 <i>y</i>	189 ± 14 <i>z</i>	
	Glutamic acid	Control	278 ± 19 <i>a</i>	557 ± 76 <i>b</i>	755 ± 100 <i>c</i>	<i>TxR</i>
		CRL	355 ± 20 <i>x</i>	925 ± 224 <i>x</i>	605 ± 107 <i>x</i>	
Amidic amino acids	Asparagine	Control	33.3 ± 2.2 <i>a</i>	75.5 ± 6.4 <i>b</i>	90.7 ± 8.5 <i>b</i>	<i>TxR</i>
		CRL	44.0 ± 2.5 <i>x</i>	138 ± 18 <i>y</i>	73.3 ± 11.5 <i>x</i>	
	Glutamine	Control	5.52 ± 0.73 <i>a</i>	17.4 ± 3.8 <i>ab</i>	22.9 ± 5.1 <i>b</i>	<i>TxR</i>
		CRL	9.05 ± 1.07 <i>x</i>	17.9 ± 3.7 <i>x</i>	11.6 ± 2.9 <i>x</i>	
Hydroxyl amino acids	Serine	Control	1377 ± 56 <i>a</i>	1594 ± 38 <i>b</i>	1167 ± 60 <i>a</i>	<i>TxR</i>
		CRL	1309 ± 31 <i>x</i>	1592 ± 151 <i>x</i>	776 ± 37 <i>y</i> ###	
	Threonine	Control	29.2 ± 2.4 <i>a</i>	65.1 ± 5.0 <i>b</i>	139 ± 18 <i>c</i>	<i>TxR</i>
		CRL	35.2 ± 4.8 <i>x</i>	95.5 ± 14.7 <i>y</i>	95.2 ± 11.3 <i>y</i>	
Aromatic amino acids	Tyrosine	Control	32.2 ± 3.9 <i>a</i>	67.6 ± 4.4 <i>b</i>	33.7 ± 6.3 <i>a</i>	<i>T</i>
		CRL	24.1 ± 3.5 <i>x</i>	72.6 ± 5.2 <i>y</i>	21.2 ± 3.7 <i>x</i>	
	Tryptophan	Control	918 ± 22 <i>a</i>	1121 ± 51 <i>b</i>	1399 ± 143 <i>b</i>	<i>TxR</i>
		CRL	1010 ± 43 <i>x</i>	1119 ± 91 <i>x</i>	1003 ± 45 <i>x</i>	
	Phenylalanine	Control	37.4 ± 1.6 <i>a</i>	22.7 ± 2.1 <i>b</i>	21.4 ± 2.6 <i>b</i>	<i>TxR</i>
		CRL	40.1 ± 1.9 <i>x</i>	39.7 ± 5.4 <i>x</i>	21.8 ± 3.9 <i>y</i>	
Other amino acids	Cysteine	Control	9.38 ± 0.31 <i>a</i>	8.95 ± 0.53 <i>a</i>	8.3 ± 0.4 <i>a</i>	<i>TxR</i>
		CRL	11.0 ± 0.5 <i>x</i>	6.69 ± 0.75 <i>y</i>	9.8 ± 0.5 <i>x</i>	
	Alanine	Control	152 ± 11 <i>a</i>	357 ± 38 <i>b</i>	428 ± 39 <i>c</i>	<i>TxR</i>
		CRL	181 ± 14 <i>x</i>	501 ± 61 <i>y</i>	275 ± 44 <i>x</i>	
	Proline	Control	156 ± 12 <i>a</i>	309 ± 19 <i>b</i>	504 ± 69 <i>c</i>	<i>TxR</i>
		CRL	167 ± 15 <i>x</i>	607 ± 105 <i>y</i>	366 ± 80 <i>x</i>	
	Glycine	Control	93.0 ± 9.4 <i>a</i>	190 ± 26 <i>a</i>	309 ± 47 <i>b</i>	<i>TxR</i>
		CRL	87.7 ± 10.1 <i>x</i>	236 ± 36 <i>y</i>	185 ± 17 <i>y</i>	
	Methionine	Control	30.6 ± 1.6 <i>a</i>	8.13 ± 1.36 <i>b</i>	6.4 ± 1.6 <i>b</i>	<i>TxR</i>
		CRL	43.2 ± 1.2 <i>x</i> #	3.74 ± 1.22 <i>y</i>	4.9 ± 1.3 <i>y</i>	
	Ornithine	Control	2.94 ± 0.31 <i>a</i>	6.80 ± 0.86 <i>b</i>	15.5 ± 3.1 <i>c</i>	<i>T</i>
		CRL	3.53 ± 0.43 <i>x</i>	9.99 ± 1.66 <i>y</i>	14.1 ± 2.0 <i>y</i>	
Sum	Total amino acids	Control	3352 ± 108 <i>a</i>	4813 ± 265 <i>b</i>	5502 ± 480 <i>b</i>	<i>TxR</i>
		CRL	3560 ± 98 <i>x</i>	5955 ± 715 <i>y</i>	3838 ± 314 <i>x</i> ##	

Data are mean ± S.E.M (n=10-11 animals per group) of control dams and moderate calorie restricted dams during lactation (CRL). Statistics: *T*, effect of lactation time; *R*, effect of maternal calorie restriction during lactation; *TxR*, interaction between the effect of lactation time and the effect of maternal calorie restriction during lactation (p<0.05, ANOVA repeated measures). Kolmogorov-Smirnov test was used for testing the normality of the distribution of the samples. Differences between CRL and control were assessed by Mann-Whitney *U* test (for no

normally distributed samples) or by Student's *t* test (for normally distributed samples) and Bonferroni correction for 21 analytes was applied: #, ## and ###, CRL vs control when $p < 0.0023$, $p < 0.0005$ and $p < 0.00005$, respectively. In both control and CRL milk samples, differences between the three days of sample collection during lactation were assessed by Bonferroni post hoc test: *a*, *b*, and *c*, for differences between day 5, 10 and 15 respectively, in control milk samples, ($p < 0.05$); *x*, *y* and *z*, for differences between day 5, 10 and 15 respectively, in CRL milk samples, ($p < 0.05$).

Figure 1 shows the concentration of free glycation adducts analyzed in milk samples at different days of lactation. Different levels were found for most of them between control and CRL dams, with the differences depending on the day of lactation. In fact an interaction between the maternal calorie restriction during lactation and the time of lactation was found for MG-H1, CEL, GH1, CMA, CML, MOLD, 3DG-H and Pyrraline adducts ($p < 0.05$, ANOVA repeated measures). Levels of all of the abovementioned adducts increased from day 5 to day 10 of lactation, in both control and CRL dams, although the increase was generally higher in CRL animals; hence CRL animals showed at day 10 of lactation higher levels of CEL, GH1, CMA, CML, 3DG-H and pyrraline than controls ($p < 0.005$, by Student's *t* test and Mann-Whitney *U* test). However, in control animals, levels of most of them (MG-H1, CEL, GH1, CMA, CML, MOLD, 3DG-H) were maintained or showed further increase at day 15 of lactation, while CRL animals showed decreased levels compared to those of 10 days. Pyrraline was an exception because their levels decreased in both control and CRL animals from day 10 to day 15 of lactation. Thus, compared to controls, CRL animals showed, at day 15 of lactation, lower levels of MG-H1 and Pyrraline.

No differences were found concerning FL and Glucosepane adducts in milk from control and CRL animals, but levels were also dependent on the day of lactation ($p < 0.05$, ANOVA repeated measures).

Figure 2 shows the concentration of free oxidation and nitration adducts in milk samples at different days of lactation. A significant interaction between the effects of maternal calorie restriction during lactation and the lactation time was observed for MetSO, Dityrosine, NFK, AASA and GSA ($p < 0.05$, ANOVA repeated measures). At day 5 of lactation, no differences were observed between the levels of the aforementioned adducts in milk from control and CRL dams. At day 10, dityrosine and AASA levels were higher in milk from CRL dams compared to that of controls ($p < 0.0002$, by Student's *t* test), but differences were not sustained thereafter. In turn, at day 15 of lactation, the free adduct NFK was present in lower levels in CRL milk in comparison to control milk ($p < 0.002$, by Mann-Whitney *U* test). Different patterns of MetSO and GSA were also observed between control and CRL dams. Both control and CRL dams showed increased levels of MetSO in milk from day 10 to day 15 of lactation, but the increase in the control group was much higher than that of CRL dams (Bonferroni post hoc test, $p < 0.05$). Concerning GSA, levels in control dams increased steadily with the day of lactation, while levels in CRL dams peaked at day 10, but levels tended to decrease thereafter. Levels of the nitration adduct 3-NT in milk samples were not different between both groups, but levels were affected by the day of lactation ($p < 0.05$, ANOVA repeated measures).

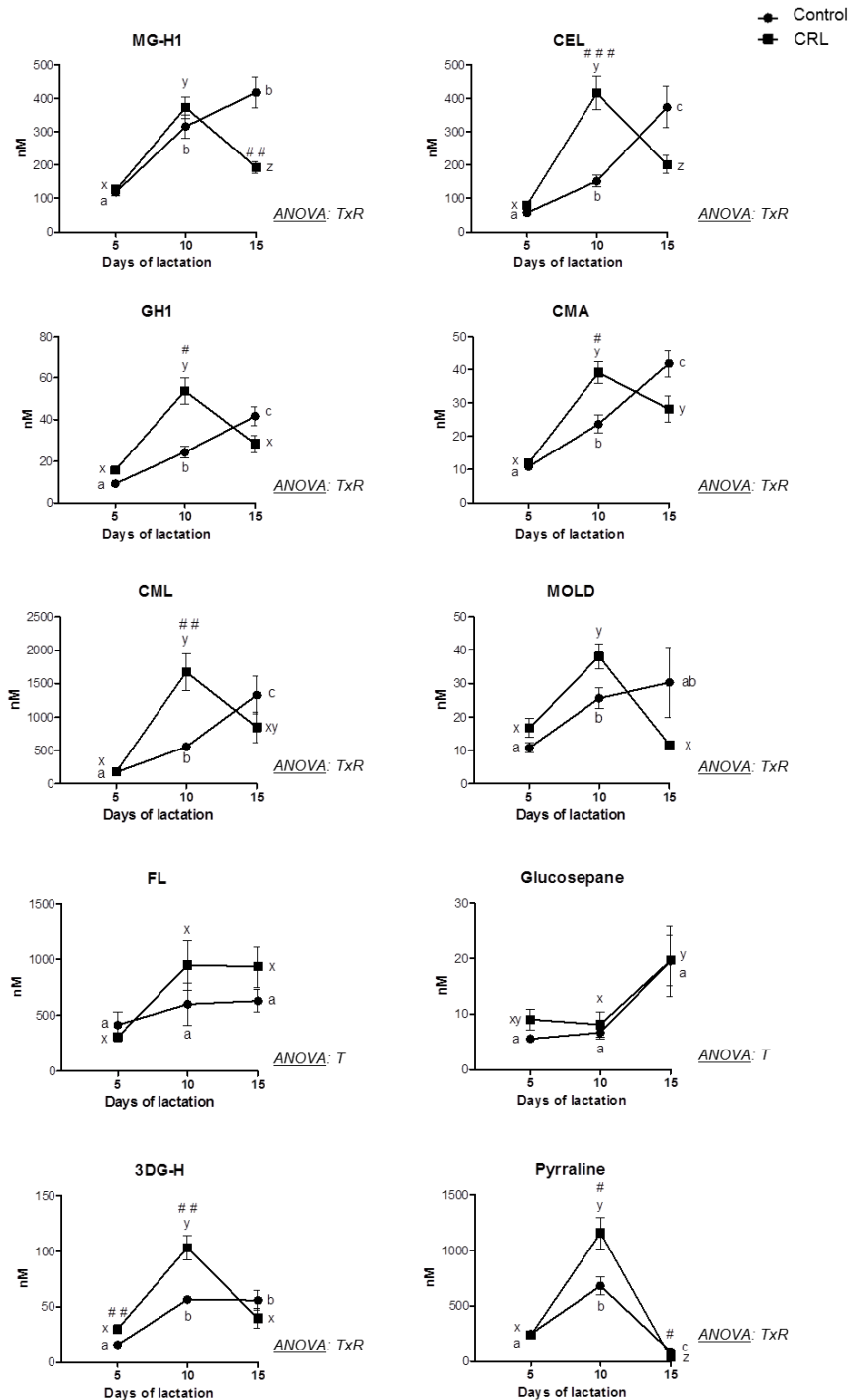


Figure 1 Concentration of free glycation adducts in the ultrafiltrate of milk samples from day 5, 10 and 15 of lactation. Data are mean \pm S.E.M (n=11 animals per group) of control dams and moderate maternal calorie restricted dams during lactation (CRL). Statistics: Kolmogorov-Smirnov test was used for testing the normality of the distribution of the samples. Differences between CRL and control were assessed by Mann-Whitney *U* test (for non normally distributed samples) or by Student's *t* test (for normally distributed samples) and Bonferroni correction for 10 analytes was applied: #, ## and ### CRL respect to control when $p < 0.005$, $p < 0.001$ and $p < 0.0001$ respectively. In both control and CRL milk samples, differences between the three days of sample collection during lactation were assessed by Bonferroni post hoc test: a, b, and c, for differences between day 5, 10 and 15 respectively, in control milk samples, ($p < 0.05$); x, y and z, for differences between day 5, 10 and 15 respectively, in CRL milk samples, ($p < 0.05$). Abbreviations: MG-H1, N δ -(5-hydro-5-methyl-4-imidazolone-2-yl)ornithine; CEL, N ϵ -carboxyethyl-lysine; GH1, N δ -(5-hydro-4-imidazolone-2-yl)ornithine; CMA, N ω -carboxymethylarginine; CML, N ϵ -carboxymethyl-lysine; MOLD, bis(lysyl) crosslink derived from methylglyoxal; FL, Fructosyl-lysine; 3DG-H, 3-deoxyglucosone.

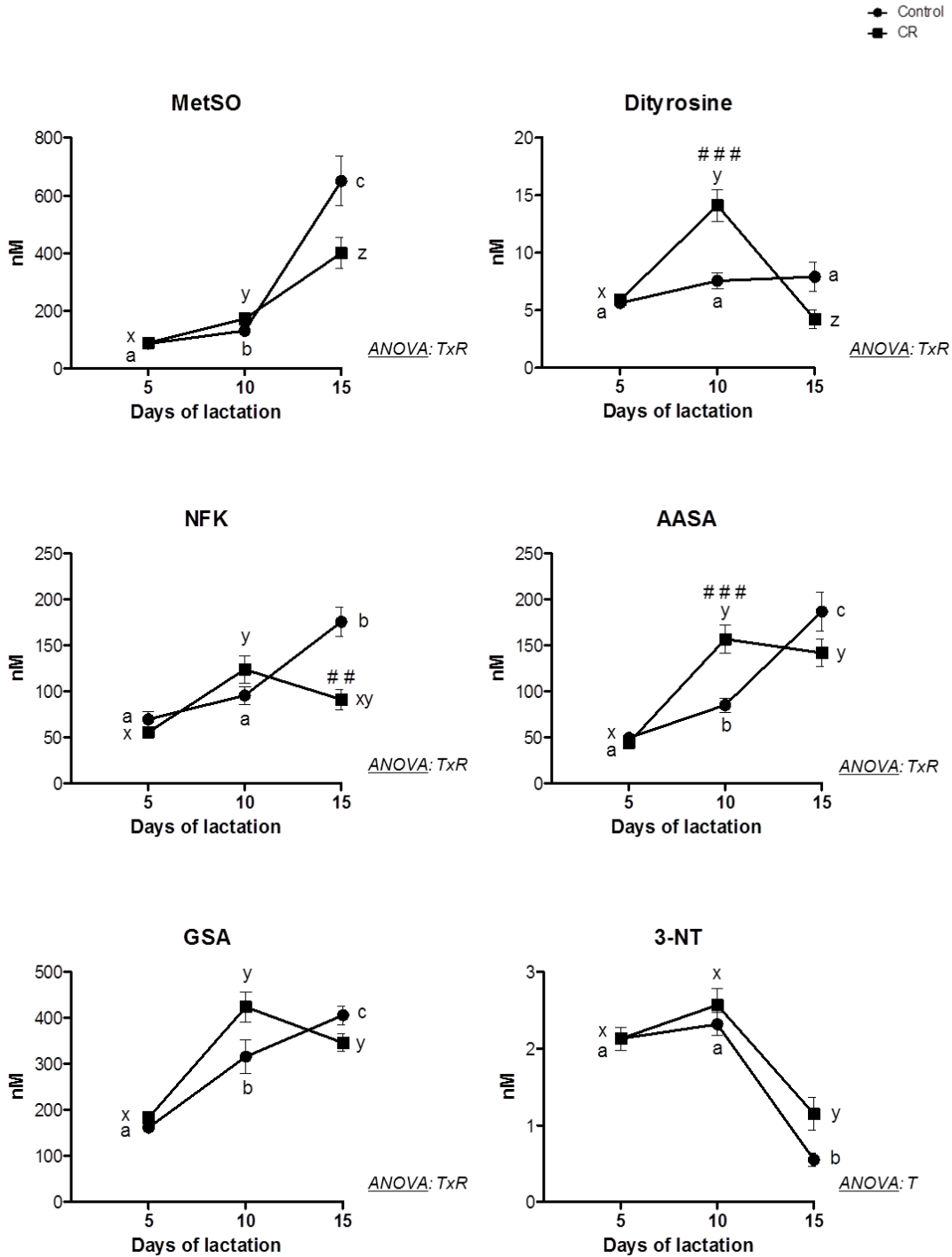


Figure. 2 Concentration of free oxidation and nitration adducts in the ultrafiltrate of milk samples from day 5, 10 and 15 of lactation. Data are mean \pm S.E.M (n=11 animals per group) of control dams and moderate maternal calorie restricted dams during lactation (CRL). Statistics: Kolmogorov-Smirnov test was used for testing the normality of the distribution of the samples. Differences between CRL and control were assessed by Mann-Whitney *U* test (for no normally distributed samples) or by Student's *t* test (for normally distributed samples) and Bonferroni correction for 6 analytes was applied: ## and ### CRL respect to control when $p < 0.001$ and $p < 0.0001$ respectively. In both control and CRL milk samples, differences between the three days of sample collection during lactation were assessed by Bonferroni post hoc test: a, b, and c, for differences between day 5, 10 and 15 respectively, in control milk samples, ($p < 0.05$); x, y and z, for differences between day 5, 10 and 15 respectively, in CRL milk samples, ($p < 0.05$). Abbreviations: MetSO, methionine sulfoxide; NFK, N-formylkynurenine; AASA, aminoacidic semialdehyde; GSA, glutamic semialdehyde; 3-NT, 3-Nitrotyrosine.

Discussion

Age-related chronic diseases, such as obesity, diabetes and cardiovascular and renal disorders, have in common elevated oxidant stress linked to a subinflammatory state (Vlassara 2005). Among other factors, cumulative glycooxidation or AGEs formation may be an important contributor to oxidant stress. In turn, a reduction of AGEs levels is associated with suppression of several immune defects, insulin resistance and diabetic complications (Chuyen 2006). Therefore, identification of ways to reduce formation of AGEs, as well as oxidation and nitration products, in biological systems may represent a potential key to reduce age-related chronic diseases. According to this, in mammals, the only major intervention that shows a reduction in oxidant stress and consequently an effectively slow of the age-related processes is calorie restriction (Vlassara 2005). In this sense, we have reported that moderate maternal energy restriction during lactation in rat dams has protective effects against overweight and related metabolic alterations in their offspring (Palou, et al. 2010a; Palou, et al. 2011; Torrens, et al. 2014). Here, it is shown that this condition during lactation in rat dams affects the concentration of free amino acids as well as AGEs and the analogous oxidation end products in maternal plasma and in milk depending on the day of lactation, hence affecting the supply of these compounds to their offspring during this critical window of development. This may be potentially related with the improved metabolic health of the offspring, although a direct cause-effect of the aforementioned compounds needs to be further elucidated.

The quantitative screening of protein damage biomarkers in maternal plasma samples at day 21 of lactation showed significantly lower levels in CRL dams of the free glycation adducts MG-H1, 3DG-H, FL, MOLD and the free oxidation biomarker AASA compared to control dams. Interestingly, when the amino acid content was measured in plasma samples of dams at weaning, we observed that almost all the amino acids determined, except aspartic acid, glutamic acid, glutamine, tryptophan, cysteine and glycine showed concentrations significantly lower in CRL dams than in controls. This is in concordance with the lower levels of free glycation adducts observed in plasma samples of CRL dams, above mentioned. Protein damage by glycation is implicated in protein misfolding. Misfolded proteins are degraded by the proteasome to ensure the high quality of biological proteins (Thornalley 2008). As a result, free amino acids and free glycation adducts are released into the bloodstream (Thornalley 2005). In this sense, as moderate calorie restriction seems to reduce glycation reactions, the number of glycated proteins formed is lower; hence, the turnover of these misfolded proteins is reduced and consequently, less free glycation adducts and less free amino acids in plasma are released into the bloodstream. This fact could explain the reduced levels of free glycation adducts and of most of the free amino acids in plasma samples from CRL dams compared to control dams.

Milk composition is known to be affected by maternal food intake (Lonnerdal 1986). The results obtained in the present study show that maternal calorie restriction during lactation has a notable impact on the content of most of the free glycation and oxidation adducts in milk, but the effects are dependent on the day of lactation. It is known that breast milk changes dynamically during breastfeeding period to accommodate the infant's needs (Klenovics, et al. 2013); here it is also shown that levels of adducts in milk also change considerably throughout lactation, in both control and CRL dams. As a general trend, free glycation and oxidation adducts levels in milk increased from day 5 to 10 of lactation, the increase being higher in CRL dams. This trend continued in milk from control dams, while it was reverted in CRL dams, thus milk from CRL dams at day 15 of lactation showed lower free adduct levels than controls. These results at day 15 of lactation are in concordance with the quantification of protein

damage biomarkers in 21-day-maternal plasma samples, in which free adducts in CRL dams were significantly lower than in control dams.

Levels of the AGEs FL and glucosepane, and of 3-NT, a marker of protein nitration, showed a similar pattern throughout lactation in control and CRL dams, but it was different compared to the rest of adducts. In turn, MetSO levels increased in both control and CRL dams throughout lactation in both groups, but the increase from day 10 to 15 was also greater in control dams. Pyrraline levels in milk also showed a different pattern throughout lactation compared to other free adducts, since levels peaked at day 10 of lactation (showing CRL milk higher levels than controls) but they decreased thereafter more markedly in CRL dams.

The factors and mechanisms responsible for the increase in some of the markers of protein damage during the first part of lactation are not known. It would appear that the onset of lactation represents a situation of stress for dams which is reflected in an increase of protein damage markers in milk. However, this situation seems to be reverted from day 10 of lactation, particularly in CRL dams. Some authors have reported that exposure to high levels of AGEs during early childhood could be a decisive event that turns on the innate immune response to a proinflammatory mode; once started, such a course is likely to result in diseases such as diabetes, at a young age (Mericq, et al. 2010). Thus, the decrease in protein damage markers found in milk from CRL dams, which is more evident for AGEs, may potentially contribute to the beneficial effects previously described in their offspring (Palou, et al. 2010a; Palou, et al. 2011; Torrens, et al. 2014).

Infant formula represents a model of AGE-rich diet. Industrial processing of infant formulas results in the formation of substantial amounts of AGEs, especially CML, hence their content in infant formulas is therefore several times greater than the content in human breast milk (Sebekova, et al. 2008). It is known that breastfeeding, compared to formula feeding, is able to bring immediate and lasting programming beneficial effects, conferring protection against obesity and related pathologies later in life (Ip, et al. 2007; von Kries, et al. 1999). Differences in nutrient composition between infant formula and breast milk, such as the higher protein content or the lack of leptin and other biologically active substances in infant formula, may play a pathophysiological role and account for the higher predisposition of formula-fed infants to later disturbances related to the metabolic syndrome (Koletzko, et al. 2005; Palou and Picó 2009; Savino and Liguori 2008). The presence of higher amount of AGEs in infant formulas compared to breast milk has also been suggested to play a role (Mericq, et al. 2010). In this regards, AGEs from infant formulas are at least partially absorbed from the gastrointestinal tract contributing to the circulating pool of AGEs. This fact may explain the reported difference in the serum levels of CML between breast-fed and formula-fed infants (Sebekova, et al. 2008). The higher amounts of AGEs in formula-fed infants may precondition to insulin resistance (Mericq, et al. 2010), although recent follow up study of breast-fed and formula-fed infants has shown that AGEs absorbed from infant formulas do not seem to be either involved in formula-consumption-associated reduced insulin sensitivity, or accompanied by enhanced oxidative stress and micro-inflammation (Klenovics, et al. 2013). Nevertheless, it should be mentioned that although infant formulas have a substantial rise of early and advanced glycation end-products compared to breast milk (Pischetsrieder and Henle 2012), they generally contain only traces of other heat-born toxic substances (Erkekoglu and Baydar 2010). It has been assumed that the negative health effects of thermally processed foods do not result from ingestion of CML *per se*, but rather from other AGEs, or additive effects of different heat-processing-derived substances (Klenovics, et al. 2013). Therefore, more studies are needed to elucidate whether the intake of high levels of AGEs in early infancy, coming from either infant formula or maternal milk, may affect postnatal programming by acting at sensitive stages during

lactation, and contribute to increased incidence of diabetes and other pathologies later in life (Klenovics, et al. 2013).

All in all, the lower content of protein damage markers in milk from CRL dams at day 15 of lactation compared to milk from control dams may be related with the lower propensity of their offspring against later metabolic diseases (Palou, et al. 2010; Palou, et al. 2011; Torrens, et al. 2014). However, the presence of greater amounts of some of them on day 10 of lactation is difficult to interpret in terms of benefits in the offspring, beyond some potential effect on protein digestion and amino acid supply. In this sense, free amino acid content in milk through lactation followed a pattern similar to that observed for some free adducts. Specifically, levels of Isoleucine, Valine, Histidine, Aspartic acid, Glutamic acid, Asparagine, Threonine, Alanine, Proline and Glycine in milk from control animals increased steadily throughout lactation, while in CRL animals, the content of these amino acids peaked at day 10 - reaching levels generally higher than those of milk from control dams - but decreased thereafter. The concentration of total amino acids in milk also followed the same trend as the aforementioned individual amino acids. It should be noted that, in rodents, the gastrointestinal tract is functionally immature for about the first 2 weeks of life, but extensive changes occurs during the third week (Morisset 1993), which might represent changes in the absorptive capacity. It is precisely at this time when pups start to eat solid food in combination with maternal milk (Oliver, et al. 2002). Therefore, differences in milk composition allowing higher supply of free amino acids during the first part of the suckling period can be considered nutritionally convenient, when digestive capacity of proteins and peptides of the pups is limited.

In conclusion, moderate maternal energy restriction during lactation in rats results in lower amount of glycation and oxidation free adducts in maternal plasma as well as in milk during the second part of lactation. Therefore, lower adduct levels in breast milk might contribute to the health benefits observed in the adult offspring of moderate energy restricted dams during lactation and could be hypothesized as potential biomarker of breast milk benefits.

Chapter V.

Recapitulation

Recapitulation

The results obtained during the development of this PhD thesis, and organized in the presented manuscripts, describe some of the effects of moderate maternal energy restriction during critical windows of development, such as the first half of pregnancy or the whole lactating period, in relation to obesity propensity and metabolic health status of their offspring. Such characterization — mainly throughout anthropometric measures, circulating parameters, gene expression analysis and histological studies — sheds light upon the potential mechanisms underlying the metabolic programming under these particular nutritional environments, and gives some clues about potential strategies to prevent or revert programmed obesity and related metabolic alterations. Remarkably, the use of these animal models has made possible to identify potential biomarkers of metabolic health that could be used as tools to determine the effectiveness of nutritional interventional strategies during critical periods of development aimed at a better protection against obesity and its related metabolic disorders in adult life.

Maternal nutritional conditions during gestation and lactation lead to differential programming on the mechanisms involved in the control of energy balance of their offspring, resulting in different outcomes in the propensity to suffer obesity in adult life. As previously shown by our group, moderate maternal calorie restriction (20%) during the first half of gestation has lasting, gender-dependent effects in the offspring. In particular, these animals are programmed for higher food intake in adulthood and show impaired ability to regulate energy homeostasis; hence, this concludes in higher body weight, but only in males. The results obtained here show that the offspring of dams that underwent a 20% calorie restriction during the first half of pregnancy (CRG) are programmed for insulin and central leptin resistance, favoring obesity development, although only manifested in males. Already at early stages of life (postnatal day 25), CRG pups, both males and females, showed in hypothalamus lower *Insr* and *Obrb* mRNA expression levels than their controls; while peripherally, in rWAT and liver, only mRNA levels of *Insr* were significantly lower in CRG animals compared with their controls. This suggests that CRG animals display early central and peripheral insulin resistance and central leptin resistance, which can be responsible for the impaired control of food intake and, hence, for the hyperphagia observed in these animals.

A possible determinant of the early detrimental effects observed in the offspring of moderate maternal calorie restriction during gestation could be an alteration in the neonatal leptin surge of these animals. The leptin surge is known as the rise of plasma leptin levels during the neonatal period (around days 9 to 10 of lactation) and is considered important for programming the structural and functional development of hypothalamic orexigenic and anorexigenic centers. In control animals, the expected peak in circulating leptin levels was observed at day 9 of lactation, while in CRG pups, the leptin surge was absent. Therefore, the detrimental effects in CRG animals by maternal calorie restriction during pregnancy could be attributed, at least in part, to the lack of the neonatal circulating leptin surge; fact that underscores the importance of leptin during lactation and the critical consequences that leptin deficiency may have during a critical period in postnatal life.

Detrimental effects by maternal calorie restriction during gestation became more apparent in the adult offspring. Gender-dependent effects were observed concerning the expression profile of key genes involved in the control of energy homeostasis. Particularly, the early differences observed at the age of 25 days, regarding hypothalamic *Obrb* mRNA levels between control

and CRG animals, were maintained at the age of 6 months but only in females. CRG females also exhibited lower *Pompc* mRNA levels than their controls, which could be closely involved in their deregulated food intake control. In addition, CRG rats, unlike controls, did not show an increase in the *Npy/Pompc* mRNA ratio under fasting conditions; this suggests that CRG animals have a decreased capacity of the CNS to sense and respond to changes in nutrient availability, also contributing to explain their higher food intake.

Regarding peripheral tissues, adult CRG animals exposed to HF-diet displayed in rWAT lower *Insr* mRNA levels (only males) and lower *Obrb* mRNA levels (both males and females) than their controls. In liver, lower mRNA levels of *Obrb* were observed in CRG male animals compared to controls but only under fed conditions. Furthermore, levels of insulin and leptin signaling-related proteins were decreased in CRG rats compared with controls, particularly under fasting conditions. Concretely, CRG female animals showed lower levels of PKC δ in rWAT compared with their controls, suggesting a decrease in insulin signaling and glucose uptake by the adipose tissue. Moreover, CRG females also displayed lower hepatic levels of total STAT3 (only under fed conditions) and of pSTAT3 compared to their controls. Conversely, CRG male animals showed higher concentration of total STAT3 in rWAT, which could be the result of their hyperleptinemia; this may allow certain leptin signaling in CRG male animals. These findings, concerning the expression levels of insulin and leptin receptors in peripheral tissues at early stages of life, together with the apparent impaired action of these hormones found in adult life, suggest that early programming of peripheral insulin resistance may be a direct consequence of fetal calorie restriction, while peripheral leptin resistance, which appears in adulthood and under HF diet might be secondary to insulin resistance or to central leptin resistance (**manuscript 1**).

Obesity has generally been considered difficult to be reverted once it is fully established, and hence, development of strategies for its prevention is considered of great interest. In this thesis we have addressed the possibility to reverse or prevent, at early ages, programmed obesity and its related metabolic alterations by increasing hepatic fatty acid oxidation (FAO) through AAV-mediated gene transference of the cDNA of *Cpt1am* —encoding for a permanently active form of CPT1A insensitive to its physiological inhibitor malonyl-CoA—. In this regard, the results obtained here show that overexpression of *Cpt1am* at early ages prevents some of the metabolic disorders associated to gestational maternal calorie restriction. According to results described in manuscript 1, adult CRG animals present features of impaired insulin and leptin sensitivity. AAV-*Cpt1am* injection reverted the alterations in plasma insulin levels, HOMA-IR, and leptin/adiponectin (L/A) ratio in CRG animals. This treatment was also able to revert, at least partially the impairment in leptin sensitivity occurring in CRG animals, as evidenced by circulating levels and hepatic *Obrb* mRNA levels.

CRG rats also displayed an increased adipocyte size in the rWAT and a trend to present greater amounts of lipid vesicles and TG content in liver, compared to control animals. Interestingly, AAV-*Cpt1am* injection in these animals resulted in a significant reduction in the adipocyte size to levels similar, or even lower, than controls, and also in a decreased number of hepatic lipid vesicles. This lower TG content in liver, which in turn may explain their relative protection to develop insulin resistance, could be associated in part to the lower expression levels of lipogenesis-related genes, such as glucose-6-phosphate dehydrogenase (*G6pd*) and Acetyl-CoA carboxylase subunit alpha (*Acaca*), in this tissue compared to their untreated controls. In addition, adult CRG animals displayed higher blood pressure, lower energy expenditure and lower locomotive activity than control rats; AAV-*Cpt1am* injection in these animals led to reverse the increased blood pressure, to partially recover the energy expenditure levels of

controls, and to restore the decreased locomotive activity. AAV-*Cpt1am* injection in CRG animals was also able to ameliorate inflammatory state, as evidenced by the lower hepatic TNF α mRNA expression levels of these animals in comparison to their controls.

These findings support the possibility to revert, prevent or ameliorate programmed propensity to develop obesity and other features of the metabolic syndrome, which have been generally considered irreversible, via a strategy addressed to increase hepatic FAO by overexpression of *Cpt1a* (**manuscript 2**).

Unlike the detrimental lasting effects of maternal calorie restriction during gestation on their offspring, we show here that moderate maternal calorie restriction during lactation protects their offspring from the development of obesity and other metabolic-related alterations, such as dyslipidemia, insulin resistance, and hyperinsulinemia, in adult life associated with HF diet feeding. Particularly, male and female offspring of dams that were exposed to 30% moderate calorie restriction during lactation (CRL) displayed lower body weight than their controls, already since the first days of life. This lower body weight was associated with lower food intake and was maintained while animals were growing, but was gradually attenuated with aging. The effect of maternal calorie restriction during lactation was more evident under HF diet feeding conditions, since the gain of body weight, accumulated body fat and also the increased intake of calories under HF-diet was lower in CRL animals than controls, especially in females. Interestingly, CRL males appeared to be more protected against HF-diet induced insulin resistance than controls; as evidenced, under HF-diet feeding conditions, by their HOMA-IR values, their higher NEFA response to fasting conditions and their lower circulating TG levels (under fed conditions) than their control. In females, no differences in HOMA-IR values were found between control and CRL animals, although neither control nor CRL rats showed increased HOMA-IR index under HF-diet. Concerning the leptin system, the protecting effect of maternal calorie restriction during lactation was especially evident under HF diet conditions. Notably, CRL females maintained their leptin levels under HF-diet feeding conditions similar to the levels of NF fed animals; this fact denotes an improvement of leptin sensitivity in these animals.

Regarding the importance of leptin supply by maternal milk during lactation, we found here that CRL dams maintained similar levels of leptin in breast milk to those of control dams. Leptin in milk comes from both the maternal blood source and its production in the mammary gland. Plasma leptin levels between control and CRL dams were not different. Concerning its production by the mammary gland, although CRL dams suffered a reduction in the weight of this tissue, they showed higher leptin mRNA and protein levels than controls, and total leptin abundance estimated in the whole mammary gland was still higher than in their controls.

All in all, these results revealed that moderate maternal calorie restriction during lactation confers certain protection of their offspring against diet-induced obesity and its related metabolic alterations, particularly insulin resistance and hyperleptinemia, although in a sex-dependent manner (**manuscript 3**).

Mechanisms underlying the developmental programming effects of maternal calorie restriction during lactation could involve changes in the expression of energy balance-related genes. Indeed, herein we found changes or adaptations at gene expression level of key genes related with central and peripheral leptin and insulin sensibility, which may explain, at least in part, the improved metabolic health in the offspring of dams exposed to moderate calorie restriction during lactation. Concretely, in adulthood, CRL male rats showed in hypothalamus higher mRNA expression levels of *Oxbr* and a tendency to lower mRNA levels of *Socs3* than controls,

while CRL females displayed decreased *Npy/Pomc* ratio (under HF-diet) and increased hypothalamic mRNA levels of *Insr* in comparison to controls. These changes at gene expression level in the hypothalamus illustrate the better central response to insulin action of CRL females and the better central leptin sensitivity of CRL males, and all in all can explain the improved feeding behavior and the higher resistance to develop diet-induced obesity of these animals. At peripheral level, CRL male animals were resistant to the decrease of mRNA levels of *Insr* and of lipogenic-related genes in rWAT under HF-diet feeding conditions, whereas their controls presented an impairment of these processes. These results suggest that CRL males have an improved response to insulin and an increased capacity to store the excess of fat in the adipose tissue. CRL females did not display changes concerning the expression levels of *Insr*; however, it is worth noting that CRL females presented higher *Obrb* and *Cpt1* mRNA levels (only under NF diet) in rWAT than their controls, suggesting that these animals have greater FAO capacity in adipose tissue, which is very important for the control of their body weight and fat reserves. In summary, these findings illustrate that maternal calorie restriction during lactation produces gender-dependent changes at gene expression level in the hypothalamus and rWAT of their offspring, programming these animals to be more resistant to the development of overweight/obesity and other metabolic-related alterations under HF-diet conditions (**manuscript 4**).

The analysis of the expression profile of genes involved in energy homeostasis in animal models less prone to develop metabolic syndrome-related alterations could be an interesting strategy to identify potential biomarkers of improved metabolic health. Most of the currently existing biomarkers are developed for disease and are used in medicine as diagnostic tools for screening, diagnosis and monitoring the disease progression. However, biomarkers derived from disease processes do not serve to assess the potential benefits of particular nutrients or changes in dietary habits. Thus, identification of early biomarkers of metabolic health is of great interest. To achieve this, we have used the animal model consisting in the offspring of moderate calorie restricted dams during lactation. Although previous studies were performed with 30% maternal calorie restriction, here we performed a more moderate restriction, 20%, because this could be more representative or applicable in humans. In this regard, here we observed that even a less severe maternal calorie restriction during the suckling period also programs their offspring for a better metabolic health, as evidenced by the lower body weight, lower adiposity and lower cumulative food intake of adult CRL animals, under HF-diet feeding conditions, in comparison to their controls (being the differences more evident in females). Moreover, concerning circulating parameters, CRL animals displayed, at early ages, increased adiponectin and decreased circulating insulin and leptin levels, in comparison to their controls; this suggests an improved insulin and leptin sensitivity. In adulthood, CRL animals also maintained improved insulin and leptin sensitivity, as evidenced by their lower values of L/A ratio and HOMA index (particularly females) in comparison to control animals. In addition, CRL animals were more protected against dyslipidemia and hepatic steatosis, and showed an improvement of the lipid handling capacity, as evidenced by the presence of lower plasma levels of TG (under HF-diet) and hepatic lipid content (under NF and HF-diet) in comparison to controls.

Notably, moderate maternal calorie restriction during lactation determines changes or adaptations in the gene expression profile of key tissues in their offspring, which are already manifested since early stages of life; this suggests their potential usefulness as early biomarkers of metabolic health. Some of these adaptations were partially maintained in adulthood and were even more evident when animals were exposed to an obesogenic environment. Concretely, CRL animals at weaning displayed higher expression levels of genes involved in insulin

signaling, *Insr* and *Irs1* (rWAT); higher expression levels of genes involved in lipid mobilization and oxidation, *Atgl* and *Ppara* (rWAT); lower expression levels of the lipogenic-related gene *Srebp1c* (liver), and higher expression levels of *Obrb* (liver) than controls. In adulthood, and under HF-diet feeding conditions, only mRNA expression levels of *Irs1* (rWAT), *Atgl* (rWAT), *Srebp1c* (only NF diet-fed males, in liver) and *Obrb* (only females, in liver) showed the same trend as was observed at weaning. Interestingly, adult HF diet-fed CRL animals presented greater *Obrb* (rWAT), *Cpt1* (only females, in rWAT) and *Irs1* (only females, in liver) mRNA levels than controls, despite no significant changes were observed at early ages. Remarkably, mRNA expression levels of some genes were also correlated with some biochemical parameters in adult animals. Concretely, mRNA levels of *Irs1* (rWAT) were negatively correlated with hepatic lipid content and with circulating TG levels; mRNA levels of *Obrb* (liver) were also negatively correlated with hepatic lipids and circulating levels of insulin under *ad libitum* feeding conditions; and mRNA levels of *Srebp1c* (liver) were positively correlated with hepatic lipid content and also with HOMA index. These associations pinpoint the potential relevance of transcript levels of these genes as early biomarkers of metabolic health (**manuscript 5**).

Extrapolation from animals into humans of the above mentioned potential biomarkers identified in liver and adipose tissue is really complex and presents some limitations. The main constraint is that tissue sample obtaining requires invasive biopsies. In this regard, PBMCs represent nowadays an attractive source for identifying biomarkers of health and also a potential way to extrapolate such biomarkers from animals into humans. This is due to the fact that they can be repeatedly collected in sufficient quantities from blood, in contrast to other tissues. Moreover, gene expression levels of PBMCs may be reflective of the gene expression patterns in liver, adipose tissue and/or muscle.

Whole-genome microarray analysis of PBMCs from weaned control and CRL pups allowed to identify 278 genes differentially expressed by effect of 20% moderate maternal calorie restriction during lactation. Concretely, immune system was the most affected process in terms of gene expression, and was followed by processes of signaling, cell turnover and transcription machinery. The expression of genes involved in metabolism of proteins, carbohydrates, and lipids, were also notably affected. Concerning lipid metabolism-related genes, *Cpt1a*, *Lipe* and *Star* were up-regulated, and *Fasn*, *Lrpl*, and *Rxb* were down-regulated in 21-day-old CRL pups *versus* control. These changes in gene expression were fully confirmed by qPCR. Among them, *Cpt1a*, *Fasn*, and *Star* emerge as particularly interesting because their expression levels in PBMCs correlated with their expression levels in adipose tissue or liver, or because their expression profile was persistent in adulthood. In particular, the expression profile of *Cpt1a* in PBMCs was correlated with that occurring in liver and adipose tissue (both at weaning and in adulthood). Concerning *Fasn*, its expression levels in PBMCs at early ages correlated with its expression levels in adipose tissue. In adulthood, this expression pattern was persistent in PBMCs but only in CRL females. The expression pattern of *Star* in PBMCs of 21-day-old CRL pups was also observed in liver samples, while in adulthood, only a trend to higher mRNA levels of *Star* was observed in PBMCs. All in all, these results reveal the possibility of using the transcript levels of concrete genes related with lipid metabolism in PBMCs as early biomarkers of metabolic health (**manuscript 6**).

The nutritional environment of dams during the lactating period may produce changes in the composition of breast milk. Such changes could include modifications in the integrity of some milk proteins through glycation, oxidation or nitration reactions, which in turn generate

misfolded proteins. Modified proteins are then degraded into free amino acids and free glycation, oxidation and nitration adducts.

Quantitative screening of free amino acids and of a comprehensive range of free glycation, oxidation and nitration adducts (protein damage biomarkers) in breast milk and plasma samples revealed the existence of different levels of these compounds between control and 20% calorie restricted dams during lactation (CRL) depending on the day of lactation. Regarding breast milk, free glycation and oxidation adducts levels increased from day 5 to 10 of lactation. This increase was greater in milk from CRL animals, which showed, at day 10 of lactation, levels of CEL, GH1, CMA, CML, 3DGH, Pyrraline, Dityrosine and AASA higher than those of controls. This trend was reverted from day 10 of lactation, and especially in CRL dams whose breast milk contained, at day 15 of lactation, lower levels of free amino acids and of MG-H1, Pyrraline and NFK than controls. Likewise, in plasma, 21-day old CRL dams also showed lower levels of free adducts and of the majority of the free amino acids studied compared to controls. It is suggested that the lower glycation and oxidation free adducts in breast milk from CRL dams during the second part of the lactating period, in comparison to milk from control dams, could be related with the higher protection of their offspring against later metabolic diseases and could be considered as potential biomarkers of breast milk benefices (**manuscript 7**).

To sum up, the results of this thesis show that moderate maternal calorie restriction during the first half of gestation in rats programs their offspring for a major propensity to develop obesity and related metabolic alterations in adulthood. Some of these programmed metabolic disturbances can be reverted by enhancing hepatic FAO at early ages. Conversely, moderate maternal calorie restriction during lactation programs their offspring for a better metabolic health and protection against obesity development through adaptations in the expression profile of energy homeostasis-related genes in key tissues, such as hypothalamus, adipose tissue and liver. Some of these adaptations were already observed at early stages of life in adipose tissue and liver. Notably, transcriptome profiling of PBMC samples revealed the possibility to use transcript levels of concrete genes as early biomarkers of metabolic health, but they need to be further validated in other animal models and, particularly, in humans. Concerning the factors potentially involved in the benefits of maternal calorie restriction during lactation, the analysis of protein damage biomarkers in milk suggests that the lower content of glycation and oxidation free adducts during the second part of the lactating period, in comparison to their controls, could contribute to the improved metabolic health in their offspring. However, a direct cause-effect of these compounds needs to be further elucidated.

Chapter VI.

Conclusions

Conclusions

1. Moderate maternal calorie restriction of 20% in rats during the first 12 days of pregnancy programs their offspring for a lower capacity to respond to insulin and to central leptin action. This may explain the hyperphagia and other metabolic alterations that show these animals in adulthood, favouring obesity development. Particularly, males show higher and earlier harmful effects than females, who appear to be more resistant to the effects of this maternal condition, in terms of maintenance of body weight, in spite of the altered gene expression profile in energy balance-related tissues (**manuscript 1**).

2. The lack of transient increase in plasma leptin levels during the suckling period, the so-called “neonatal leptin surge”, in the offspring of calorie restricted dams during gestation appears to be closely associated with the adverse health effects observed in these animals in adulthood (manuscript 1).

3. Increased hepatic fatty acid oxidation (FAO) at a juvenile age, through injection of AAV-*Cpt1a* vectors (adeno-associated virus containing the mutant form of carnitine palmitoyltransferase 1a), reverses or prevents some of the metabolic disorders observed in offspring of rat dams subjected to gestational calorie restriction when exposed in adulthood to obesogenic conditions, such as impaired insulin and leptin sensitivity, and increased systolic blood pressure. This treatment is also able to improve inflammatory markers, decrease hepatic triglyceride content, and restore the locomotive activity that was decreased in these animals in comparison to controls. Therefore, enhancement of hepatic FAO at early ages could be considered as a strategy for reverting metabolic disturbances related to adverse developmental programming (**manuscript 2**).

4. Unlike calorie restriction during gestation, moderate calorie restriction of 30% in rat dams during lactation has lasting beneficial effects on their offspring. Particularly, these animals become less prone to diet-induced obesity and related metabolic alterations in adult life, such as dyslipidemia, insulin resistance and hyperleptinemia (**manuscript 3**).

5. The offspring of 30% calorie restricted lactating rat dams (CRL) show enhanced sensitivity to insulin and leptin signalling, particularly evident under HF-diet feeding conditions. This effect is associated to gender-dependent adaptations at gene expression level in hypothalamus and visceral adipose tissue. Particularly, CRL males seem to be more protected against HF-diet induced peripheral insulin resistance, leading to an improved capacity to store the excess of fat in the adipose tissue. They also show improved capacity to respond to leptin at the central level. In turn, CRL females appear to be programmed for a better sensitivity to the peripheral actions of leptin on the adipose tissue, and display a better capacity to respond to insulin at central level (**manuscript 4**).

6. A less severe food restriction (20%) during lactation in rat dams, which could be more easily representative or applicable in humans, also programs the offspring for a better metabolic health in terms of body weight and lipid handling capacity. These effects may be related to early adaptations in the gene expression profile in WAT and liver, affecting lipogenic and oxidative capacity and increasing their sensitivity to the peripheral effects of leptin and insulin, which suggests a better control of energy metabolism. Some of these adaptations were partially maintained in adulthood, but were more evident when animals were exposed to an obesogenic environment. Among genes that exhibit changes at the expression level in early

ages, insulin receptor substrate (*Irs1*) in rWAT and the long form of the leptin receptor (*Obrb*) and the sterol-regulatory-element-binding protein (*Srebp1c*) in the liver are of relevance because their transcript levels in adult animals were associated with hepatic lipid content and circulating parameters, such as triacylglycerides and insulin, and insulin resistance indexes; thus, they could be considered as potential biomarkers of a healthy phenotype (**manuscript 5**).

7. Whole-genome transcriptome profiling of peripheral blood mononuclear cells (PBMCs) samples from the offspring of moderate (20%) calorie restricted dams during lactation allowed the identification of 278 known genes differentially expressed with respect to controls. Among lipid metabolism-related genes, carnitine palmitoyltransferase 1a (*Cpt1a*), fatty acid synthase (*Fasn*) and steroidogenic acute regulatory protein (*Star*) emerge as particularly interesting because their expression levels in PBMCs correlated with their expression levels in adipose tissue or liver, or because their expression profile was persistent in adulthood. These findings reveal the possibility of using transcript levels of the above mentioned genes in PBMCs as early biomarkers of metabolic health, which may also provide a valid biological readout for the study of metabolic processes in humans (**manuscript 6**).

8. Moderate calorie restriction (20%) in rat dams during lactation results in diminished amounts of markers of protein damage by glycation and oxidation in maternal plasma and in milk during the second part of lactation, hence affecting the supply of these compounds to their offspring. The lower presence of protein damage markers in milk may contribute to the health benefits observed in the offspring, while these compounds might be hypothesized as potential biomarkers of this condition (**manuscript 7**).

Conclusiones

1. Una restricción calórica materna moderada del 20% durante los 12 primeros días de gestación en ratas programa a la descendencia para una menor capacidad de respuesta a la insulina y a la acción central de la leptina. Esto explicaría la hiperfagia y otras alteraciones metabólicas que presentan estos animales en edad adulta, favoreciendo a su vez el desarrollo de obesidad. En concreto, los machos sufren efectos nocivos más acentuados y con mayor anticipación que las hembras, que parecen ser más resistentes a los efectos de esta condición materna en relación al mantenimiento del peso corporal, a pesar de mostrar un perfil de expresión génica alterado en tejidos relacionados con el balance energético (**manuscrito 1**).

2. La ausencia de un incremento transitorio en los niveles plasmáticos de leptina durante el periodo de lactancia, conocido como “pico de leptina neonatal”, en las crías de ratas sometidas a una restricción calórica durante la gestación, parece estar estrechamente relacionado con los efectos adversos para la salud observados en estos animales en edad adulta (manuscrito 1).

3. El incremento de la oxidación hepática de ácidos grasos en edades tempranas, mediada por la inyección de vectores AAV-*Cpt1a*m (virus adeno-asociado conteniendo una forma mutante de la carnitina palmitoiltransferasa 1a), revierte o previene algunas de las alteraciones metabólicas observadas en las crías de ratas sometidas a una restricción calórica gestacional cuando se exponen a un ambiente obesogénico en edad adulta, tales como la menor sensibilidad a la insulina y a la leptina, y el aumento de la presión arterial sistólica. Este tratamiento también mejora los niveles de marcadores de inflamación, descende el contenido de triglicéridos hepáticos y es capaz de restaurar la actividad locomotora que estaba disminuida en estos animales en comparación con los controles. Por lo tanto, el aumento de la oxidación de los ácidos grasos en el hígado, en etapas tempranas de la vida, podría considerarse una estrategia para revertir alteraciones metabólicas relacionadas con una programación adversa durante el desarrollo (**manuscrito 2**).

4. A diferencia de la restricción calórica durante la gestación, una restricción calórica moderada del 30% en ratas madre lactantes, tiene efectos beneficiosos a largo plazo sobre su descendencia. Particularmente, estos animales son menos propensos a desarrollar obesidad inducida por la dieta así como alteraciones metabólicas relacionadas en edad adulta, tales como la dislipidemia, resistencia a la insulina e hiperleptinemia (**manuscrito 3**).

5. Las crías de ratas sometidas a una restricción calórica del 30% durante la lactancia (CRL) presentan una mayor sensibilidad a la señalización de la insulina y la leptina, especialmente patente en condiciones de alimentación con una dieta hiperlipídica (HL). Este efecto se asocia a adaptaciones dependientes del sexo en el perfil de expresión génica en el hipotálamo y en el tejido adiposo visceral. En concreto, los machos CRL parecen estar más protegidos frente al desarrollo de resistencia periférica a la insulina inducida por una dieta HL, lo que determina una mayor capacidad para almacenar el exceso de grasa en el tejido adiposo blanco. También muestran una mejorada capacidad para responder a la leptina a nivel central. Por otro lado, las hembras CRL parecen estar programadas para una mayor sensibilidad a las acciones periféricas de la leptina en el tejido adiposo, y presentan una mejor capacidad para responder a nivel central a la insulina (**manuscrito 4**).

6. Una restricción calórica menos severa (20%) durante la lactancia en ratas madre, que puede ser más fácilmente representativa o aplicable a humanos, también programa a la descendencia para una mejor salud metabólica, en cuanto al peso corporal y a la capacidad para el procesamiento de los lípidos. Estos efectos podría estar relacionado con adaptaciones tempranas en el perfil de expresión génica en el TAB y el hígado, afectando la capacidad lipogénica y oxidativa e incrementando su sensibilidad a los efectos periféricos de la leptina y la insulina, lo que sugiere un mejor control del metabolismo energético. Algunas de estas adaptaciones se mantuvieron parcialmente en edad adulta, pero fueron más evidentes cuando los animales se expusieron a un ambiente obesogénico. Entre los genes que exhibieron cambios a nivel de expresión génica en edades tempranas, son destacables el sustrato 1 del receptor de insulina (*Irs1*) en el TAB y la forma larga del receptor de la leptina (*Obrb*) y la proteína de unión al elemento regulador de esteroides (*Srebp1c*) en el hígado, porque sus concentraciones de transcritos en edad adulta se asociaron con el contenido hepático de lípidos y con parámetros circulantes, tales como triacilgliceroles e insulina, así como con indicadores de resistencia a la insulina; por lo tanto, podrían ser considerados como potenciales biomarcadores de fenotipo saludable (**manuscrito 5**).

7. El análisis transcriptómico de muestras de células mononucleares de sangre periférica (PBMCs) de crías de madres sometidas a una restricción calórica moderada del 20% durante la lactancia, permitió la identificación de 278 genes conocidos que se expresan diferencialmente con respecto a las controles. Entre los genes relacionados con el metabolismo lipídico, el de la carnitina palmitoiltransferasa 1a (*Cpt1a*), la ácido graso sintasa (*Fasn*) y la proteína de regulación esteroideogénica aguda (*Star*) destacan como particularmente interesantes, bien porque sus niveles de expresión en PBMCs se correlacionaron con sus niveles de expresión en el TAB o el hígado, o bien porque su perfil de expresión se mantuvo en edad adulta. Estos resultados revelan la posibilidad de utilizar la concentración de transcritos de los genes anteriormente mencionados en PBMCs como biomarcadores tempranos de salud metabólica, los cuales podrían proporcionar una potencial herramienta biológica válida para el estudio de procesos metabólicos en humanos (**manuscrito 6**).

8. La restricción calórica moderada (20%) durante la lactancia en ratas determina una menor concentración de marcadores de daño proteico por glicación y oxidación en el plasma materno y en la leche durante la segunda mitad de la lactancia, afectando por tanto al suministro de estos compuestos a sus crías. La menor presencia de marcadores de daño proteico en la leche podría contribuir a los beneficios sobre la salud observados en la crías de estos animales, a la vez que estos compuestos podrían ser propuestos como posibles biomarcadores de esta condición (**manuscrito 7**).

Chapter VII.

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