UNIVERSIDAD DE LAS ISLAS BALEARES

TESIS DOCTORAL

ESTUDIO EN RATAS DE LA INFLUENCIA DE LA LACTOSA Y LA LEUCINA DURANTE LA LACTANCIA SOBRE LA DESCENDENCIA

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Estudio en ratas de la influencia de la lactosa y la leucina durante la lactancia sobre la descendencia

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La doctoranda

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A mis padres y
a mi hermana

Caminando en línea recta,
no se puede llegar muy lejos
(El Principito)

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INDICE

ABREVIATURAS

A-myb	Factor de transcripción MYB (Mieloblastosis)
ACACA	a- Acetil Co-A carboxilasa
ADPN	Adiponectina
AR	Receptor de andrógenos
ATP	Adenosín trifostato
Bcl2	Célula B CLL/Linfoma 2
BMP	Proteína morfogenética del hueso
c-Met	Receptor del factor de crecimiento de hepatocitos
CEBPs	Proteínas estimulantes de unión a CCAAT
CIDEA	Cell death-inducing DFFA-like effector a
CREB	Proteína de unión al elemento de respuesta a AMPc
DHA	Ácido docosahexanoico
EFG	Factor de crecimiento epidérmico
FADs	Desaturasas ácidos grasos
FASN	Ácido graso sintasa
FGF	Factor de crecimiento de fibroblastos
FIL	Factor inhibidor de la lactancia
GLI3	GLI *family zinc finger 3*
GLUT	Transportador de glucosa
HBV	Virus de la hepatitis B
HGF	Factor de crecimiento hepatocitario
Hoxc6	Homeobox 6
Hoxc9	Homeobox 9
IgA	Inmunoglobulina A
IGF	Factor de crecimiento insulínico
IgG	Inmunoglobulina G
IgM	Inmunoglobulina M
IL-1	Interleucina 1
IL-6	Interleucina 6
IMC	Índice de masa corporal
INSIG 1	Gen inducido por insulina

L-alba	α- Lactalbúmina
LC-PUFA	Ácidos grasos poliinsaturados de cadena larga
LDL	Lipoproteína de baja densidad
LEF-1	Factor de unión 1 al potenciador linfoide
LPL	Lipoproteín lipasa
miRNA	micro ARN
MMP	Metaloproteinasas de matriz
NF-kB	Factor nuclear potenciador de cadenas ligeras κ de células B activadas
NNP	Nitrógeno no proteico
NRG	Neuroregulinas
p53	Citocromo p53
PPAR	Receptores activados por proliferadores de peroxisoma
PTH	Hormona paratiroidea
PTHrP	Proteína relacionada con la hormona paratiroidea
RT-PCR	Reacción en cadena de la polimerasa con transcriptasa inversa
SCD	Enzima estearoil-coA desaturasa
SGP2	Glicoproteína sulfatada 2
SLC25A1	Familia 25 de transportadores de solutos (miembro1)
SLC27	Familia 27 de transportadores de solutos
SREBP	Proteína de unión al elemento de respuesta a esteroles
STAT	Proteína transmisora de señal y activadora de la transcripción
TAG	Triacilglicéridos
Tbx-3	Proteínas T-box
TGF	Factor de crecimiento transformante
TNF-a	Factor de necrosis tumoral alfa
UCP1	Proteína desacopladora tipo 1
UDP	Uridina difosfato
VIH	Virus de la inmunodeficiencia humana
WNT	Proteína wingless
XOR	Xantina oxidoreductasa

RESUMEN

La lactancia materna tiene un importante papel sobre el crecimiento de la descendencia y constituye un momento crucial en su programación metabólica, modulando la propensión a determinadas enfermedades en edad adulta. La hipótesis central de desarrollo esta Tesis es que la nutrición, particularmente en etapas tempranas del desarrollo, modula y/o condiciona la susceptibilidad a la obesidad en edad adulta y con ello condiciona la expresión génica en tejidos clave. El objetivo de la presente Tesis ha sido profundizar en el conocimiento de los efectos de dos nutrientes, la lactosa y la leucina, durante la lactancia. La leche es producida por la glándula mamaria, cuyos genes son susceptibles de modulación. Una mejor comprensión de su metabolismo y de su respuesta a la dieta materna es un aspecto clave para optimizar la composición de la leche y así contribuir al óptimo desarrollo del lactante. En este contexto se han planteado dos aproximaciones experimentales:

- *El desarrollo y aplicación de un método de transfección in vivo en la glándula mamaria de rata para conseguir la sobre-expresión del gen de la* β*-galactosidasa y posibilitar la producción de una leche reducida en lactosa in situ.* La transfección ha permitido una reducción transitoria de lactosa en leche (un 35%) y galactosa (un 41%), durante los primeros 6 días, que luego recupera los valores control. La caracterización del impacto de dicha sobre-expresión sobre el metabolismo ha mostrado un aumento en la masa grasa de las madres y obesidad en edad adulta en la progenie.

- *La suplementación de la dieta materna con L-leucina durante la lactancia y el análisis de su influencia sobre la expresión génica en glándula mamaria y sobre el potencial "brite" del adiposo de la progenie*. Los resultados indican que dicha suplementación provoca cambios importantes en el metabolismo de la glándula mamaria que afecta a la expresión de genes representativos tanto de la capacidad de

transporte (Glut1, se reduce un 72%), como de la síntesis de proteína (la expresión de caseína disminuye el 50%) y de genes relacionados con el metabolismo lipídico. Es destacable que también se reduce en la expresión del gen responsable de la síntesis de lactosa (35%) que repercute en un contenido reducido de lactosa en leche (25%). Por otra parte, se ha caracterizado el potencial de expresión de marcadores de células "brite" en tejido adiposo de la progenie adulta. Los resultados muestran un dimorfismo sexual en dicha capacidad, mostrando los machos un mayor potencial "brite" respecto al que presentan las hembras. Además se ha puesto en evidencia que la intervención nutricional temprana ha modulado el potencial "brite" en la progenie adulta de manera relevante, mientras que el acceso a una dieta hiperlipídica en edad adulta muestra un menor impacto sobre dicho potencial.

En definitiva, el desarrollo de esta Tesis ha permitido poner de manifiesto la relevancia del metabolismo de la glándula mamaria así como la influencia de la dieta materna en su modulación y las repercusiones asociadas que se producen en el fenotipo de la progenie en edad adulta. Este conocimiento es necesario para definir intervenciones específicas desde etapas tempranas de la vida que contribuyan a la prevención de la obesidad.

LISTADO DE PUBLICACIONES

El trabajo realizado en la presente tesis ha dado lugar a la preparación de los siguientes manuscritos:

I. Servera M, López N, Zamanillo R, Picó C, Palou A, Serra F. *Nutrigenomics and Breast Milk, Perspectives in Obesity,* en: "Lactation: Natural Processes, Physiological Responses and Role in Maternity". Editores Lisa M. Reyes, Douglas C.O Editor, Nova Biomedical (Novapublishers), New York, 2012. ISBN 978-1-62257-265-6 (eBook)

II. Servera M, Serra F, Palou A. *Suckling low-lactose milk is associated with higher fat deposition in rat's adulthood*. Manuscrito en vías de publicación

III. Servera M, López N, Serra F, Palou A. *Impact of maternal L-leucine supplementation on mammary gland and milk composition in rats*. Manuscrito en vías de preparación

IV. Servera M, López N, Serra F, Palou A. *Expression of brite adipocytes biomarkers show gender differences and the influence of early dietary exposure.* Manuscrito en vías de publicación

I. Introducción

1. LA GLÁNDULA MAMARIA

La glándula mamaria es una estructura glandular ubicada en el pecho. Está formada por tejido conjuntivo, grasa, tejido, y posee un elevado riego sanguíneo. Se encuentra adherida al músculo pectoral mayor. Su función está ligada a la reproducción ya que es el órgano encargado de producir la leche materna.

1.1 LA GLÁNDULA MAMARIA COMO TEJIDO DINÁMICO

La glándula mamaria es uno de los órganos más asombrosos y complejos de la biología humana. Sufre cambios estructurales y funcionales durante la pubertad, la gestación, la lactancia e incluso en la etapa postmenopáusica; al igual que tiene y mantiene la capacidad de remodelarse y regenerarse. Ello le confiere esa característica dinámica, siendo uno de los órganos, si no el que más cambios sufre a lo largo de la vida.

Histológicamente, la glándula mamaria es prácticamente igual en todas las especies y está formada por un parénquima glandular y un estroma de soporte. El parénquima es un conjunto de lóbulos, integrados por una serie de conductos que terminan en unos alvéolos o acinos revestidos por epitelio, mientras que el estroma está constituido por tejido conectivo laxo con abundantes fibras de colágeno, un infiltrado linfocitario y una red de capilares. Ambos están separados por la membrana basal.

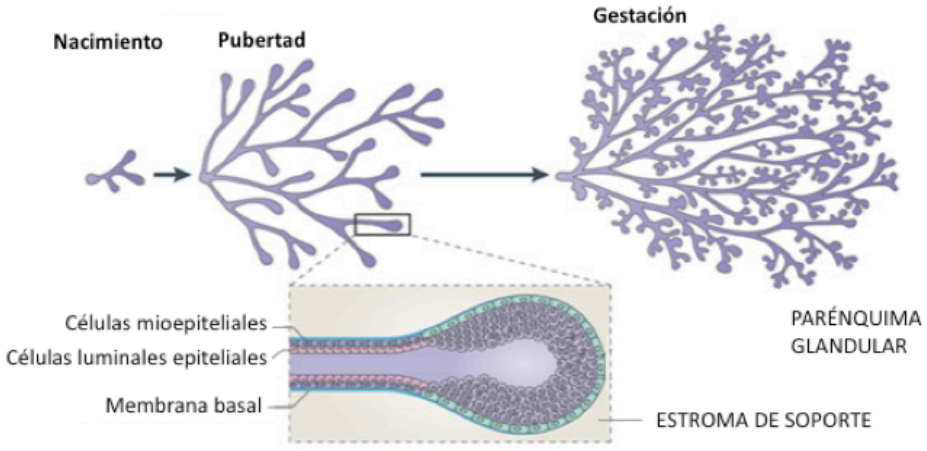

Figura 1: Evolución de la glándula mamaria

El desarrollo de la glándula mamaria ha sido minuciosamente descrito por Russo como un proceso de invasión progresiva del estroma mamario por parte del parénquima glandular,

cuyos extremos se van diferenciando progresivamente dando lugar a los diferentes tipos de lóbulos (RUSSO and RUSSO 2004; GJOREVSKI and NELSON 2011). Este proceso se da en tres etapas: la embrionaria, la puberal y la adulta.

La formación de la mama comienza en la etapa embrionaria, de manera que al nacer, ya está formado un sistema rudimentario de conductos. Cada conducto principal va ramificándose progresivamente para acabar en las unidades funcionales de esta glándula de secreción externa, que no es más que una glándula sudorípara modificada. Después del nacimiento la glándula mamaria sigue creciendo, al igual que el resto de los tejidos del organismo. En la pubertad se produce el principal crecimiento y diferenciación lobular. Se origina una proliferación del epitelio y el crecimiento del estroma que da lugar a la mama adulta cuyo desarrollo depende fundamentalmente de las hormonas ováricas (RUSSO and RUSSO 2004; MALLER *et al.* 2010; GJOREVSKI and NELSON 2011) . En la mujer adulta la glándula está formada por lóbulos tipo 1, 2 ó 3, dependiendo de la edad y del hecho de ser o no nulípara.

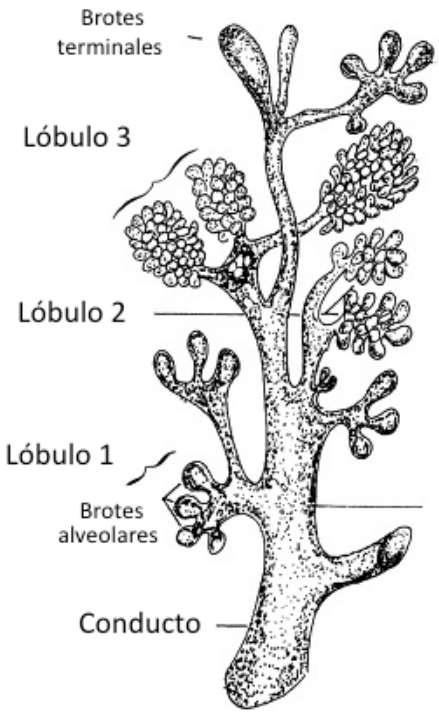

Figura 2: Tipos de lóbulos

Durante la gestación la glándula mamaria sufre su mayor desarrollo, en dos fases distinguidas: la de crecimiento y proliferación que se da en los primeros meses de gestación, y

la de la actividad secretora que se da al final. En este período los lóbulos evolucionan hacia el tipo 4. Antes y en el momento del parto se produce un activación de la mitosis y un incremento de la cantidad total de ADN en la glándula mamaria. Durante la lactancia, no se dan grandes cambios morfológicos, tan sólo un aumento en el tamaño lobular por la actividad lactogénica. La acumulación láctea o el vaciamiento de la glándula mantienen la secreción de leche durante la lactancia. Posteriormente se produce la regresión postlactacional de la glándula que consiste en una autolisis celular, fagocitando y desintegrando los lóbulos y dando lugar a una regeneración final del tejido. Durante la menopausia se produce una regresión de la glándula mamaria, que dará lugar a un mayor número de lóbulos tipo 1.

La gonadotropina, la prolactina y las hormonas sexuales controlan todos estos procesos con el objeto de asegurar su correcto desarrollo y el mantenimiento de sus estructuras así como la funcionalidad de la glándula, particularmente durante la lactancia (NEVILLE *et al.* 2002; SVENNERSTEN-SJAUNJA and OLSSON 2005; OAKES *et al.* 2008; MACIAS and HINCK 2012), si bien en la etapa fetal la regulación hormonal es prácticamente inexistente. Otras señales reguladoras de la morfogénesis mamaria, bastante menos conocidas son producidas por macrófagos, eicosanoides o algunos factores de crecimiento (STERNLICHT *et al.* 2006; MCNALLY and MARTIN 2011) que, al contrario de las hormonas, ejercen un control local. Recientemente, también se ha descrito que otras moléculas como los miRNA están implicadas también en el desarrollo de la glándula al estar presentes en el estroma y actuar sobre determinadas metaloproteinasas (MMP) que pueden regular la cantidad de colágeno en el mismo (UCAR *et al.* 2010).

Este proceso de crecimiento y diferenciación y los mecanismos que lo regulan están controlados por diferentes genes. El estudio de la expresión génica de la glándula mamaria permite indagar en primera instancia sobre los procesos que modulan y regulan la adaptabilidad de este tejido según sus necesidades y comprender mejor todas sus posibilidades funcionales.

1.2 EXPRESIÓN GÉNICA DE LA GLÁNDULA MAMARIA

1.2.1 Genes relacionados con el desarrollo de la glándula

Cada uno de los diferentes estados fisiológicos de la glándula mamaria supone cambios en la expresión génica y en la síntesis proteica. La última década ha sido testigo de una revolución en el conocimiento de las señales moleculares que regulan el desarrollo de la glándula mamaria gracias a la implementación de nuevas técnicas para la secuenciación masiva y para el abordaje del estudio proteómico completo de la leche (lactoma) o de los ARNm asociados. Así por ejemplo, se ha podido extraer el ARN contenido en los glóbulos de grasa de leche humana, lo que ha permitido estudiar de forma exhaustiva la expresión de genes en las células epiteliales de la glándula durante la lactancia (MANINGAT *et al.* 2009); así mismo, el uso de técnicas como los microarrays, la RT-PCR o la inmunohistoquímica, permite ampliar el conocimiento molecular de las adaptaciones de la glándula mamaria en diferentes especies para llevar a cabo su principal función, la lactancia (NØRGAARD *et al.* 2008; PATEL *et al.* 2011). Además, con el objeto de dilucidar los posibles mecanismos asociados a la inducción y desarrollo del cáncer de mama o la metástasis se han descrito muchos de los procesos biológicos que materializan estos cambios.

La caracterización de la modulación de la expresión génica en la glándula mamaria es compleja y específica de las diferentes etapas del desarrollo:

- **De la etapa embrionaria a la niñez**: Cowin y colaboradores en el 2010 publican una revisión sobre los procesos moleculares que ocurren en el desarrollo de la glándula mamaria durante la etapa embrionaria. En él recalcan la importancia de determinadas vías de señalización y los genes que las regulan, como la ruta Wnt, FGF o BMP, y el papel de determinados genes clave como Tbx-3, ciertos factores de transcripción, GLI3, las beta-cateninas, el receptor de IGF-1 u Hoxc6, y la regulación hormonal que tiene lugar, así como las señales que desencadenan (HENS and WYSOLMERSKI 2005; COWIN and WYSOLMERSKI 2010). A pesar que durante la embriogénesis mamaria se expresan multitud de genes tanto en el epitelio como en el estroma, para la morfogénesis de la glándula mamaria se requieren sobretodo el LEF-1, según se ha observado en ratones (BORAS-GRANIC *et al.* 2006), la proteína relacionada con la hormona tiroidea (PTHrp) (HIREMATH *et al.* 2012) y el receptor tipo 1 de la PTH (FOLEY *et al.* 2001). Éstos últimos son los responsables del dimorfismo sexual que promueve la expresión del receptor de andrógenos (AR) (DUNBAR *et al.* 1999; COWIN and WYSOLMERSKI 2010).

- **Pubertad:** Durante la pubertad, el factor de crecimiento de los hepatocitos (HGF) y su receptor c-Met induce el crecimiento ductal (BERDICHEVSKY *et al.* 1994; NIRANJAN *et al.* 1995; SORIANO *et al.* 1995). Este proceso es dependiente de las integrinas β1 funcionales (un tipo de moléculas de adhesión) , así como las lamininas, que desempeñan un importante papel en el desarrollo y diferenciación celular y por ello intervienen en la formación de tumores (KLINOWSKA *et al.* 1999). Pero el desarrollo de los ductos depende también de genes como TGF, EFG o IGF-1 (SILBERSTEIN 2001). En cuanto al crecimiento lobulillo-alveolar propio de esta etapa, cabe destacar el papel de la vitamina D, a través de su receptor (NARVAEZ *et al.* 2001) y las neurorregulinas (NRG), miembros de la familia EFG de factores de crecimiento (NIEMANN *et al.* 2000). Las MMP tienen un papel clave en este proceso de morfogénesis en combinación con genes como el TNF-α (SHEA-EATON *et al.* 2001) e IGF (FOWLKES *et al.* 1995; MARSHMAN and STREULI 2002; HINCK and SILBERSTEIN 2005). Por otra parte la dieta puede influir en el crecimiento glandular a este nivel alterando los mecanismos de crecimiento ductal o parenquimatoso, por ejemplo aumentando los efectos proliferativos del EFG (ABOU-EL-ELA *et al.* 1988; WELSCH and O'CONNOR 1989; BANDYOPADHYAY *et al.* 1993; MACLENNAN and MA 2010; LUIJTEN *et al.* 2013).

- **Embarazo:** Como se observa en la pubertad, las NRG se expresan en el estroma de la glándula mamaria durante la gestación (YANG *et al.* 1995). Ciertos genes de la familia de TGF-β también intervienen en la modulación del desarrollo glandular en esta etapa (SORIANO *et al.* 1998; SILBERSTEIN 2001; BUSSMANN *et al.* 2004). Y, determinadas moléculas de señalización como los factores de transcripción A-myb, STAT5, CREB, Hoxc9 y la ciclina D1 juegan un importante papel en el desarrollo mamario (SICINSKI and WEINBERG 1997; TOSCANI *et al.* 1997; CHEN and CAPECCHI 1999; GRONER 2002). De manera similar a lo que ocurre en la pubertad también la expresión de MMP contribuye al desarrollo del tejido (URIA and WERB 1998; HINCK and SILBERSTEIN 2005).

- **Lactancia**: La principal función de la glándula es la producción y secreción de leche para la correcta nutrición del recién nacido. Los cambios que suceden en la madre, afectan indudablemente a la leche que secreta y la glándula mamaria sufre un profundo cambio para ser funcionalmente activa en la lactancia. Por interacciones

célula-célula se produce el completo desarrollo lobulillo alveolar y la síntesis láctea. Las alteraciones en la expresión de determinadas cadherinas (que controlan la adhesión celular) en las células epiteliales influyen en este proceso (KNUDSEN and WHEELOCK 2005; NELSON 2008). El control de la síntesis láctea, tanto de la secreción como de la frecuencia, ocurre en gran parte gracias a un mecanismo de regulación autocrino por parte de un factor intrínseco de la leche: el inhibidor de la lactancia por retroalimentación (FIL, del inglés *lactation inhibitor factor*), que ejerce este control de forma local. Además los receptores hormonales también juegan un papel importante en esta fase de secreción de la leche (WILDE *et al.* 1995; WILDE *et al.* 1998). En el destete se produce la involución de la glándula mamaria, que consiste en un proceso de apoptosis de las células secretoras y epiteliales mediante muerte celular programada y la degradación proteolítica de la matriz extracelular y la membrana basal de la glándula por parte de enzimas como las MMP. Hay un gran número de genes involucrados en este proceso. FASN, p53, IL-6, SGP2, y sobretodo STAT3 ejercen una regulación positiva, mientras que STAT5, IGF (I y II) y Bcl2 lo hacen negativamente (LUND *et al.* 1996; TORRES *et al.* 2011; WATSON and KREUZALER 2011). En la primera fase la arquitectura de la glándula no cambia (es la fase reversible), mientras que en la segunda fase de involución es cuando se produce la degradación de las estructuras, se activa la adipogénesis y la posterior remodelación de la glándula mediante un proceso inflamatorio y fagocítico gracias a la activación de proteasas (que no ocurre inmediatamente después del destete). El factor de crecimiento EFG juega un papel importante en esta última fase, además el factor NF κB origina señales inflamatorias, controladas también por STAT3 (WATSON 2006).

- **Menopausia:** Poco se conoce acerca de los mecanismos reguladores de la glándula mamaria que tienen lugar en esta etapa. El epitelio sufre apoptosis, es reemplazado por grasa y el tejido intralobulillar es sustituido por colágeno. La mayoría de los estudios que se han llevado a cabo en la glándula mamaria en este período son para esclarecer los posibles mecanismos de la carcinogénesis.

Así, el desarrollo de la glándula mamaria pasa por una serie de drásticos cambios en composición, arquitectura y funcionalidad, marcados por la expresión de genes que caracterizan los diferentes estados fisiológicos del desarrollo de la misma con el principal objetivo de llevar a cabo su función: ser el órgano de producción de leche en el momento del

nacimiento. Queda patente la capacidad de la glándula a sufrir modificaciones y adaptaciones moleculares según la expresión de unos u otros genes, y que esta plasticidad la hace susceptible de sufrir alteraciones y cambios a nivel transcriptómico que pueden también deberse a agentes externos y ser de vital importancia durante la lactancia.

1.2.2 Genes relacionados con el metabolismo y producción de leche

La expresión de genes relacionados con el metabolismo y con la producción de leche en la glándula mamaria, determina al final la composición y por tanto la calidad de la misma. Aunque el estudio molecular de la glándula en humanos es difícil por los problemas éticos y prácticos para la obtención de tejido mamario durante la lactancia, se han utilizado cultivos celulares de tejido mamario y también muestras de leche para estudiar moléculas fácilmente detectables en ésta. Así, se han publicado numerosos estudios sobre linfocitos, macrófagos, otras células inmunitarias propias de la leche materna (NISHIMURA 2003), u hormonas que son secretadas en la leche y que permiten hacer una aproximación de la regulación hormonal de la glándula. Por ejemplo, se sabe que la hormona de crecimiento, la leptina, la prolactina y la progesterona regulan la secreción de leche en la glándula mamaria. (NEVILLE *et al.* 2002). Bien es cierto que en animales, particularmente en aquellas especies que producen leche para el consumo humano debido al interés que tiene para la industria, se conoce con más detalle los aspectos relacionados con la regulación de la expresión génica determinantes de la cantidad y calidad de la leche, y en muchas ocasiones ello permite hacer una aproximación a lo que ocurre en la especie humana.

En los últimos años se ha profundizado en el conocimiento de los mecanismos que modulan la biosíntesis de *lípidos* y la regulación de la lipogénesis (ANDERSON *et al.* 2007) La glándula mamaria es uno de los órganos lipogénicos más importantes en prácticamente todas las especies de mamíferos, ya que la mayoría de los lípidos producidos durante la lactancia son secretados a la leche. De entre los genes lipogénicos expresados en glándula mamaria cabe destacar los transportadores de ácidos grasos (familia SLC27), la acetil CoA carboxilasa (ACACA) cuya expresión varía también en tejido adiposo durante la lactancia, así como enzimas importantes en el manejo de ácidos grasos como la ácido graso sintasa (FASN), la desaturasa (SCD), y la LPL, al igual que alguno de los ya mencionados como relevantes también en algunas fases del desarrollo, como el PPARγ o el SREBP (BARBER *et al.* 1992b; HAN *et al.* 2010; HAN *et al.* 2012). Hay que tener en cuenta sin embargo, que estas rutas o la

expresión de algunos de estos genes pueden verse alterados por intervenciones dietéticas en la madre; ya que por ejemplo, ante una dieta rica en grasa la disponibilidad de ácidos grasos para la síntesis de triglicéridos por la glándula es mayor y por tanto la regulación de esta síntesis se modula a nivel postranscripcional, promoviendo cambios en la expresión de la ácido sintasa (FASN), el gen inducido por insulina INSIG1, el transportador mitocondrial SLC25A1 o la SCD2 (RUDOLPH *et al.* 2010). En cuanto a la secreción de lípidos desde la glándula a la leche, se han identificado algunos genes relacionados con este transporte como la xantina oxidoreductasa (XOR), fuertemente influenciada por la expresión de CIDEA, el CIDEA propiamente dicho, las adipofilinas, que pertenecen a la familia de las perilipinas (proteínas asociadas a adiposomas o cuerpos lipídicos), e incluso la lipoproteína de baja densidad LDL ha sido objeto de estudio intenso por su papel en el transporte de lípidos en la glándula mamaria (MONKS *et al.* 2001; MARTÍNEZ *et al.* 2002; CHONG *et al.* 2011; WANG *et al.* 2012).

La síntesis de *lactosa* (uno de los principales componente de la leche) tiene lugar a partir de la glucosa o la UDP-galactosa, gracias a la lactosa sintasa y la proteína específica de la glándula α-lactalbúmina (L-alba). Una vez más todos estos procesos están altamente regulados por estimulación hormonal (RUDOLPH *et al.* 2007; MOHAMMAD *et al.* 2012).

Sin embargo, en la glándula mamaria ocurren , además de la síntesis de lípidos y lactosa muchos otros procesos metabólicos durante la lactancia, que también modulan en último término la composición de la leche. En primer lugar los nutrientes deben llegar al tejido mamario, para ello son necesarios transportadores específicos, que están minuciosamente regulados para asegurar la correcta disponibilidad de los mismos en el proceso de síntesis láctea. Entre éstos los más importantes son los transportadores de glucosa, los de aminoácidos y los de ácidos grasos. Todos ellos se ven aumentados durante la lactancia. Por otro lado, la glándula mamaria experimenta cambios en la expresión de genes relacionados con la mayoría de las rutas anabólicas y catabólicas, como la glicólisis, el ciclo de las pentosas fosfato, la gluconeogénesis, el ciclo del ácido cítrico, la síntesis de triglicéridos o la de colesterol. Por último están los genes reguladores de todos estos procesos, como la familia de CEBPs, el IGF, PPAR, STAT, WNT o SREBP. Algunos de ellos ya se han mencionado por su papel en el desarrollo de la glándula mamaria (RUDOLPH *et al.* 2007). Por último los genes responsables de los ritmos circadianos en otros tejidos también han sido recientemente descritos en glándula mamaria. Estos genes coordinan desde los cambios metabólicos y hormonales necesarios para el inicio de la lactancia como la expresión de otros

genes que también siguen una pauta cíclica a lo largo del día, con diferentes picos de expresión (CASEY *et al.* 2009; MANINGAT *et al.* 2009) .

2. EL PRODUCTO DE SECRECIÓN DE LA GLÁNDULA MAMARIA: LA LECHE

2.1. COMPOSICIÓN DE LA LECHE

Como se ha comentado, es durante el embarazo y la lactancia cuando se produce la plena madurez de este órgano y se dan los cambios más significativos que aseguran la síntesis y secreción de leche correctamente y en la cantidad demandada por el recién nacido; esta leche debe ser el alimento que aporte todos los nutrientes necesarios para su desarrollo, crecimiento y protección a enfermedades, y por ello, tiene una composición bioquímica y celular específica, por lo que la composición de la leche es propia de cada especie (HASSIOTOU and GEDDES 2013).

En el embarazo la glándula mamaria se prepara para llevar a cabo la síntesis y secreción de la leche materna. Para ello, y como se ha visto, posee el engranaje que le permite sintetizar y secretar una leche que cubra todas las necesidades nutricionales de las crías, ya que se convierte en su único alimento durante las primeras etapas de la vida.

Gracias a la inhibición hormonal la secreción de leche permanece inactiva hasta el momento del parto. Entonces, la lactancia se estimula activamente por el instinto de succión de las crías y los cambios hormonales que se dan en la glándula. La prolactina estimula el inicio de la secreción por los acinos glandulares que provocan la congestión sanguínea, posteriormente el calostro y por último la leche madura. La oxitocina es fundamental para el mantenimiento de la lactancia.

Al instaurarse la lactancia incrementa el volumen de sangre, el ritmo cardíaco y se movilizan nutrientes hacia la glándula en detrimento de otros tejidos, ya que es en este período en el que se produce la mayor demanda de nutrientes por parte del tejido mamario, incluso más que en el embarazo (WILLIAMSON *et al.* 1995; BUTTE *et al.* 1999; HERRERA 2000; PICCIANO 2003). El estado nutricional y fisiológico de la madre influye considerablemente en esta utilización de sustratos (BARBER *et al.* 1992a; RASMUSSEN 1992; FLINT and VERNON 1998).

La glucosa es la mayor fuente de energía en la producción de leche, utilizada para la síntesis de lactosa y lípidos (los dos componentes más abundantes en la leche humana) (PATEL *et al.* 2011; ZIVKOVIC *et al.* 2011), aunque la glándula mamaria también está programada para utilizar triglicéridos que provienen de otros tejidos (NEVILLE and PICCIANO 1997).

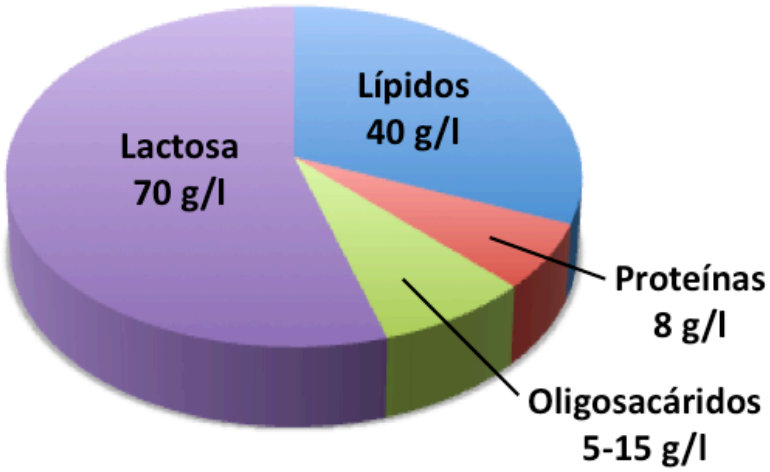

Figura 3: Composición de macronutrientes en la leche materna humana

La leche es un producto elaborado por la glándula mamaria y no un simple filtrado o concentrado del plasma sanguíneo. Aunque el desarrollo de la mama y el mecanismo de producción de leche son similares en todas las especies, la composición de la leche varía de unas a otras y el suministro de leche está adaptado a las exigencias de cada una.

Mediante la leche la madre suministra nutrientes, inmunidad y componentes reguladores del crecimiento al recién nacido. En la especie humana, en los primeros días postparto, cambia el epitelio mamario y se incrementan los niveles de leche de 30 a 150 ml por día, hasta alcanzar los 300 ml entre el quinto y sexto día postparto. Estos incrementos en la cantidad de leche van unidos a los cambios en su composición. La primera leche o calostro, se produce hasta el quinto día; la leche de transición, del sexto día al 16º, y la leche madura, que es la definitiva, se instaura a partir del día 17. En la lactancia prolongada, se ha descrito un tipo de leche con una composición algo diferente, con más lactosa, proteínas y minerales.

La leche humana experimenta cambios importantes en su composición, desde el calostro hasta la leche definitiva. Algunos autores, como Neville han descrito que el aumento del volumen lácteo va precedido por una disminución significativa de sodio, cloro y proteínas, y un aumento de lactosa. Entre el segundo y quinto día de lactancia, la producción de leche abundante significa también un aumento de glucosa, fosfatos y calcio, y una disminución del pH (AGUILAR CORDERO 2005) .

En la producción de la leche humana la glándula sintetiza sus constituyentes de la leche y los cede al lumen glandular. La leche se obtiene por síntesis en las propias células mamarias o bien por el transporte desde el plasma hasta esas células. Cada célula mamaria interviene como una unidad elaboradora de leche. Todas las células secretoras funcionan como una unidad y producen todos los compuestos de la leche. La secreción láctea es continua, aunque la evacuación de la glándula es discontinua y no todos los alvéolos funcionan de forma sincrónica durante la secreción. La leche humana tiene la misma presión osmótica que el plasma; sin embargo se diferencia en algunos compuestos respecto a otras especies, ya que la concentración de los principales iones monovalentes es menor, y el nivel de lactosa, mayor.

El mecanismo de secreción de la leche por el cual pasa a la luz alveolar puede ser, según la sustancia, por:

- **Difusión:** Agua e iones monovalentes
- **Exocitosis:** partículas de proteínas y ciertos carbohidratos
- **Secreción apocrina**: Glóbulos de grasa
- **Pinocitosis**: Inmunoglobulinas
- **Vía paracelular**: utilizada por las células para llegar a la leche

El *calostro* producido los primeros días tendrá unas 54 kcal/100ml, con 2,9 g/100 ml grasa, 5,7 g/100 ml lactosa y 2,3 g/100ml de proteínas (3 veces más proteína que la leche madura). Se destaca su alta concentración en IgA y lactoferrina junto a su contenido en linfocitos y macrófagos (100,000/mm^3), lo que le confiere una acción protectora al lactante ya que las células no son destruidas en su aparato digestivo. Entre las vitaminas liposolubles destaca el β-caroteno (responsable del color amarillento).

Entre el calostro y la leche madura se produce una leche de *transición,* cuya composición está entre una y otra, como su propio nombre indica, y va variando con el transcurso de los días.

La leche *madura* es la que sintetiza la glándula a partir de los 10 días después del parto y hasta el final de la lactancia.

2.1.1. Composición de la leche madura

La leche madura contiene 70 kcal/100ml, su volumen promedio es de 700 ml/día en los 6 primeros meses postparto, descendiendo a unos 500ml/día en el 2º semestre. Sus principales componentes son: proteínas, minerales, hidratos de carbono, grasas, agua y vitaminas. La leche contiene un 88% de agua con una osmolaridad semejante al plasma (286 mosm).

La lactosa es su principal *carbohidrato* (7,3 mg %) y la principal fuente energética del lactante (glucosa + galactosa). La galactosa es utilizada en la síntesis de galactolípidos, de gran importancia en el desarrollo del sistema nervioso central (SNC) del niño. Otros carbohidratos complejos se encuentran libres o unidos a proteínas como la N-acetilglucosamina y oligosacáridos. También los hay que proceden de la hidrólisis de la lactosa (PARK and OH 2010) y han sido identificados como estimulantes del desarrollo del lactobacilo que protege al lactante en el intestino.

La *parte proteica* (0,9 g/100ml) está compuestas por caseína (30%) y proteínas del suero (70%), como lactoferrina, lactoalbúmina, lisozima, IgA, IgG, IgM y albúmina. Contiene proteínas de baja concentración como: enzimas, moduladores del crecimiento y hormonas. La lactoalbúmina tiene un alto valor biológico y es la más abundante, la lactoferrina favorece la absorción del Fe en el intestino y además posee acción bacteriostática. La lisozima tiene actividad antiinflamatoria y la IgA es la principal inmunoglobulina de la leche humana. La leche además posee un alto contenido de nitrógeno no proteico (NNP), entre el 20 y el 30% del N total, siendo el principal componente la urea que es utilizada por el lactante como fuente de N. Los aminoácidos libres como la taurina, son necesarios para conjugar los ácidos biliares y como posible neurotransmisores. Los aminoácidos esenciales como la leucina, son de

especial importancia para asegurar el contenido proteico e influyen en la homeostasis de la energía en las crías (LÓPEZ *et al.* 2010; LEI *et al.* 2012)

Las *grasas* aportan el 50% de las calorías de la leche, siendo el componente más variable. Su concentración es menor al inicio que al final de la toma del lactante y la calidad de los ácidos grasos se ve especialmente afectada por la dieta materna (JUAN *et al.* 2000).

Composición de la leche materna

Componentes	Leche humana	Calostro	Leche de vaca
Agua (%)	88	87	88
Energía (kcal %)	70	58	69
Lactosa (g %)	7,3	5,3	4,8
Nitrógeno total (mg %)	171	360	550
Nitrógeno proteico (mg %)	129	313	512
Nitrógeno no proteico (mg %)	42	47	32
Proteínas totales (g %)	0,9	2,3	3,3
Caseína (g %)	0,25		2,73
α-lactoalbúmina (g%)	0,26	0,16	0,11
β-lactoalbúmina (g%)	0	0	0,36
Lactoferrina (g%)	0,17	0,33	Trazas
Lisozima (g%)	0,05		Trazas
Ig A (g %)	0,14	0,36	0,003
Grasas totales (g %)	4,2	2,9	3,8
Ácido linoleico (% grasas)	8,3	6,8	1,6
Colesterol (mg %)	16	28	
Calcio (mg %)	28		125
Fósforo (mg %)	15		96

Las fórmulas lácteas comerciales para alimentar a los recién nacidos se elaboran en base a leche vacuna modificada (maternizadas) y son carentes de algunos de los elementos bioactivos de la leche humana. Ningún sustituto tiene igual aporte nutritivo en calidad,

cantidad y proporciones de sus componentes, con la misma biodisponibilidad para el crecimiento y desarrollo, ni con menor riesgo de desnutrición infantil (BALLARD and MORROW 2013).

Lactosa

Como se ha comentado la lactosa es el componente mayoritario de la leche y la principal fuente de energía para el lactante. La lactosa es un disacárido formado por glucosa y galactosa, que define la cantidad de agua de la leche. El alto contenido en lactosa determina las deposiciones blandas del lactante que permite la absorción del Ca en el colon. Pero además de ser el principal azúcar de la leche (JENSEN 1995), la lactosa adquiere especial interés porque en individuos adultos puede dar lugar a un síndrome de malabsorción conocido como intolerancia a la lactosa (TUNICK 2009). Las leches con menor contenido en lactosa mejoran los síntomas, pero en el caso de la leche materna las estrategias para conseguir una leche de estas características son complejas. Se han utilizado por ejemplo ratones transgénicos que expresan una enzima que hidroliza la lactosa en la glándula mamaria *in vivo* (JOST *et al.* 1999b). Mediante la reducción del complejo de la "lactosa sintasa" también se ha conseguido una leche con contenido reducido en lactosa, pero en este caso se produce una reducción tan marcada de azúcares provoca una elevada viscosidad en la leche (STINNAKRE *et al.* 1994; L'HUILLIER *et al.* 1996a; L'HUILLIER *et al.* 1996b). La intolerancia a la lactosa genera en muchas ocasiones la disminución drástica en el consumo de leche y los datos que se conocen hasta el momento sobre la influencia de la leche materna en este síndrome y las posibilidades de mejora con actuaciones a edades tempranas son escasos.

Leucina

La leucina es uno de los aminoácidos esenciales de cadena ramificada presentes en la leche y necesarios para la síntesis de proteína en la misma. Además son precursores de otros aminoácidos de la leche que se sintetizan en la glándula mamaria y que se pueden encontrar de forma libre en la leche. La oxidación de estos aminoácidos en la glándula mamaria proporciona muchos de los sustratos necesarios para el metabolismo de la glándula, como el transporte de nutrientes, la síntesis de lactosa o de lípidos. Además, la leucina puede activar determinadas vías del tejido mamario que aumentan la síntesis de proteína e influye en la homeostasis energética en las crías (LÓPEZ *et al.* 2010; LEI *et al.* 2012) y se le ha atribuido

también un papel protector ante la obesidad y otras enfermedades metabólicas (LAYMAN and WALKER 2006).

2.2. FACTORES QUE DETERMINAN EN LA COMPOSICIÓN DE LA LECHE

Las condiciones medioambientales, el estado nutricional y fisiológico de la madre y su alimentación, pueden influir directa o indirectamente en la regulación de genes en la glándula mamaria que determinan la composición de la leche. Las variaciones en la composición de la leche pueden promover en las crías el desarrollo de enfermedades metabólicas como la obesidad o la resistencia a insulina, en edad adulta (PALOU and PICÓ 2009). Bien es cierto que estas modificaciones no comprometerían el crecimiento normal de las crías, pero sí la propensión a determinadas enfermedades.

La nutrición de la madre afecta no sólo al desarrollo de la glándula mamaria durante la gestación como ya se ha mencionado, sino también a la leche, en cuanto a su producción y su composición, e incluso en animales domésticos se han descrito algunos de los efectos que pueden producirse sobre la descendencia (VAN DER LINDEN *et al.* 2009) . En los humanos también se sabe que determinados factores maternos, como la composición corporal o la alimentación de influyen en la leche y por tanto en el desarrollo de las crías. El conocimiento sobre estos procesos es aún escaso, ya que otros factores podrían afectar al crecimiento del recién nacido, no sólo la lactancia, pero sí sabemos que los cambios que experimenta la madre pueden modificar la composición de la leche, y que este hecho influye en mayor o menor grado en las crías. Por ejemplo, el sobrepeso durante la primera semana de vida, está relacionado con el riesgo a desarrollar obesidad, así como un aumento en la adiposidad (STETTLER *et al.* 2005; TAYLOR *et al.* 2005), o una restricción calórica moderada durante la lactancia da lugar al aumento de expresión de algunos genes en la glándula mamaria, como la leptina, hormona de relevante importancia en la leche materna por su papel protector a la obesidad (PALOU *et al.* 2011).

La glándula mamaria sin embargo posee plasticidad y capacidad de minimizar las fluctuaciones que se dan en la madre, y por ello no siempre se puede definir una asociación clara entre la dieta de la madre y la composición de macronutrientes de la leche (COWARD *et al.* 1984; DEWEY 1998; VILLALPANDO and DEL PRADO 1999; QUINN *et al.* 2012). Sin embargo, sí que la desnutrición durante la lactancia puede influir en el peso corporal de la

descendencia, principalmente asociado a cambios en la concentración de lípidos y proteínas en la leche. El aporte de proteínas en la dieta de la madre lactante es crucial para el óptimo desarrollo de las crías. En estudios animales se ha descrito que las dietas hipoproteicas en madres lactantes originan un desarrollo deficiente de la glándula mamaria con la consecuente disminución de proteínas en la leche, lo cual podría estar asociado con el desarrollo de resistencia a la insulina en la edad adulta. Por otro lado, cuando se dan dietas hipocalóricas a las madres la leche tiene mayor contenido lipídico y por tanto las crías tienden a ser más obesas en la edad adulta (DE MOURA and PASSOS 2005) .

La suplementación en la dieta de la madre con otros nutrientes que son importantes en la leche también tiene consecuencias en el desarrollo de la progenie. Por ejemplo, los ácidos grasos omega 3 y omega 6 deben estar en la correcta proporción, y suplementos de LC-PUFA en la dieta de la madre influye en última instancia sobre la masa corporal de la descendencia. El ácido docosahexanoico (DHA) es muy importante biológicamente, ya que presenta altas concentraciones en el cerebro y la retina y se acumula con facilidad en tejidos neuronales durante la infancia. Por lo tanto, la leche tiene que suministrar las debidas cantidades de DHA, puesto que puede ser un elemento crucial en el desarrollo neurológico y de la capacidad visual en etapas tempranas. La cantidad de DHA en la leche varía considerablemente según la ingesta de la madre, y de hecho, el suplemento de DHA en madres lactantes provoca aumentos en la cantidad de este ácido graso en leche (MAKRIDES *et al.* 1996; JENSEN *et al.* 2000; BRENNA *et al.* 2007; JENSEN and LAPILLONNE 2009; MUHLHAUSLER *et al.* 2010; MUHLHAUSLER *et al.* 2011).

En cuanto a micronutrientes, el ácido fólico destaca por ser otro de los elementos clave en el desarrollo de las crías. Es necesario para la síntesis de ADN, y esto cobra importancia en las etapas de crecimiento y diferenciación, además es en esta fase donde pueden darse los cambios epigenéticos que en parte determinen el desarrollo de la descendencia (PICÓ *et al.* 2012). Sin embargo, el ácido fólico es tan importante para las crías que prácticamente no está influenciado por el estatus de folato que posea la madre, excepto en casos de deficiencia (LAMERS 2011). En el caso del Zn, otro micronutriente importante en la leche, la regulación de la glándula mamaria actúa en sentido similar y la concentración del mismo en la leche se mantiene al margen de los niveles de la madre (MOORE *et al.* 1984; KREBS 1998; DÓREA 2012). La existencia de transportadores en la glándula mamaria permite que los niveles en

leche se mantengan constantes, como ocurre con los aminoácidos libres (DESANTIAGO *et al.* 1998).

Cabe destacar otro ejemplo de influencia de los hábitos de la madre sobre la leche: las drogas, fármacos, tóxicos o algunos productos naturales. Si bien es cierto que las sustancias exógenas ingeridas por la madre suelen aparecer en leche a concentraciones más bajas que en plasma, es de vital importancia caracterizar los efectos que provocan y establecer las concentraciones seguras para el niño, ya que éste no dispone de los mecanismos adultos para metabolizar estas sustancias (BERLIN and BRIGGS 2005; ILETT and KRISTENSEN 2005; SERVERA *et al.* 2012)

3. INFLUENCIA DE LA LECHE SOBRE LOS LACTANTES

La salud y el crecimiento de los neonatos dependen de la producción y composición de la leche materna. Los aspectos beneficiosos atribuidos a la leche materna, que han sido avalados por numerosas evidencias científicas han originado que de un tiempo a esta parte se haya promovido la lactancia materna como el único modo único de alimentación en los primeros meses de vida, y pueden clasificarse en dos grandes grupos:

- **Los beneficios relacionados con situaciones fisiológicas**: En el crecimiento, donde la leche materna puede influir en el desarrollo cognitivo, mental y neurológico, físico y psicomotriz, en los aspectos psicosociales, en el desarrollo dental y en la respuesta inmunitaria;
- **Los beneficios relacionados con la salud**: ya que protege a la descendencia de padecer ciertas enfermedades en edades tempranas y a largo plazo (PALMER 2011). Se ha profundizado en el estudio de la lactancia materna por ejemplo, por ser beneficiosa en dermatitis atópica, por mejorar el pronóstico de cuadros infecciosos (respiratorios, gastrointestinales y del oído medio), enterocolitis, leucemia, en la infección por el VIH, el HBV (virus de la hepatitis B), y en edad adulta sobre enfermedades cardiovasculares, el nivel de colesterol, obesidad, resistencia a la insulina y diabetes tipo 1.

La leche materna contiene no sólo los macro y micronutrientes necesarios en la alimentación del lactante, sino también compuestos bioactivos, factores de crecimiento,

factores inmunológicos y compuestos que son potencialmente activos en la protección de la salud del neonato y le otorgan los mencionados aspectos beneficiosos (BALLARD and MORROW 2013).

Se ha constatado por tanto, que la lactancia materna frente a la alimentación con leches de fórmula, elaboradas a partir de leche de vaca (a la que se le igualan los contenidos de nutrientes y añaden algunos compuestos activos), es más completa y más adecuada para la alimentación de los neonatos. Así, la alimentación del neonato con leche de fórmula, se ha relacionado con sobrepeso e incluso obesidad en edad adulta, como se ha visto en varios estudios en humanos a largo plazo, y en modelos en los que la lactancia materna se prolongaba durante más o menos tiempo (DEMMELMAIR *et al.* 2006; LI *et al.* 2008; MCCRORY and LAYTE 2012). En estudios animales, Palou y colaboradores han identificado a la leptina como elemento responsable en la protección frente al desarrollo de obesidad en edad adulta y su carencia en leches de fórmula se ha vinculado a la propensión a la obesidad que se halla en edad adulta en humanos que han sido alimentados con dichas leches (PICO *et al.* 2011) (PATEL *et al.* 2009). La nutrición en edades tempranas tiene efectos a corto y largo plazo sobre la descendencia.

Por otra parte, la leche materna es susceptible de adaptar su composición a las características maternas y medioambientales, al igual que en la glándula mamaria, y como se ha señalado, puede repercutir no solo en el crecimiento y desarrollo del lactante sino también en las características de su metabolismo a largo plazo. Así pues, las modificaciones fenotípicas asociadas que se instauran en edades tempranas bajo la influencia de la nutrición en etapas perinatales configuran lo que se denomina *programación metabólica*. El concepto de programación metabólica se ha definido como "la inducción, supresión o alteración en el desarrollo de una estructura somática permanente o el ajuste de un sistema fisiológico, por parte de un estímulo temprano o agravio en un período sensible, que resulta en consecuencias funcionales a largo plazo" (LUCAS 1991).

Los primeros estudios que hacían referencia a la programación metabólica, se basaban en los aspectos descriptivos del efecto de la nutrición en edades tempranas sobre la salud en edad adulta. Sin embargo, recientemente ya se realizan estudios de intervención para esclarecer de forma concreta el impacto de la dichas intervenciones sobre la salud de la descendencia a largo término (LUCAS 1998).

Hay estudios que demuestran aspectos negativos, por ejemplo como el consumo de algunas drogas o fármacos; o positivos, como la suplementación de algún nutriente en la alimentación, pero en todo caso, se refieren a etapas cruciales de la vida como la gestación o la lactancia. El concepto de programación temprana es un término amplio que no solo hace referencia a los nutrientes, sino a cualquier compuesto que incida significativamente sobre el potencial de desarrollo del nuevo individuo (hormonas, metabolitos o neurotransmisores, entre otros). Por ejemplo, en ratas, la testosterona secretada por los testículos del feto en períodos críticos programa al cerebro hacía un comportamiento masculino en edad adulta (ANGELBECK and DUBRUL 1983), ciertas drogas teratogénicas tienen efectos a largo plazo en el desarrollo, o también la administración de fenobarbital en ratas después del parto tiene efectos en el tiempo sobre el citocromo P450 que manifiestarn posteriormente.

En este contexto, la programación nutricional hace referencia a como la nutrición en etapas perinatales puede tener efectos sobre la salud a largo plazo. Los estudios animales han aportado información relevante acerca de las potenciales consecuencias en humanos. Se dispone de evidencias en modelos animales de que cambios en la dieta en momentos perinatales modulan el desarrollo del sistema inmunitario (PÉREZ-CANO *et al.* 2012), o favorecen la aparición del síndrome metabólico, pueden promover la propensión a padecer obesidad, influyen en el crecimiento y resistencia a la insulina, diabetes (HERRERA *et al.* 2006), alteran la presión sanguínea (BARKER *et al.* 1993), y tienen efectos sobre la aterosclerosis, el comportamiento o el aprendizaje (LEWIS *et al.* 1986; DESAI *et al.* 1996; HALES *et al.* 1996; LUCAS *et al.* 1996). En *humanos*, se ha estudiado la influencia de la alimentación sobre la vista (DZIECHCIARZ *et al.* 2010), la función cognitiva y el desarrollo neuronal (GRANTHAM-MCGREGOR 1995), la resistencia a insulina (GERSTEIN 1994) o la aterosclerosis (FALL *et al.* 1992).

Los *mecanismos* biológicos para "memorizar" a lo largo de la vida la exposición nutricional en etapas tempranas y su expresión en edad adulta incluye cambios adaptativos en la expresión génica y la proliferación o adaptación celular en los tejidos programados (que en la lactancia es sobretodo la glándula mamaria). Estos mecanismos de programación nutricional pueden clasificarse en:

- **Remodelación tisular:** El modo más sencillo de ejercer efectos sobre la fisiología, el metabolismo y la salud es mediante la alteración morfológica del

tejido, ya sea en el número o el tipo de células. Esta remodelación podría ocurrir como resultado de una perturbación en la proliferación o diferenciación celular en momentos clave del desarrollo. Todos los tejidos y órganos derivan principalmente de una línea celular embrionaria progenitora. Estas líneas celulares se diferencian en el feto y primeras etapas de la vida, por lo que una falta o exceso de nutrientes y señales durante ese desarrollo puede tener consecuencias importantes.

- **Intercambio materno-fetal:** La placenta es más que un órgano de intercambio de nutrientes entre la madre y el feto. Las señales a nivel endocrino que se dan entre la placenta y el feto y entre la madre y la placenta juegan un papel fundamental en el correcto desarrollo del feto y el reparto de nutrientes (GODFREY 2002)
- **Mecanismos epigenéticos:** Las modificaciones epigenéticas que se dan en la expresión génica de los diferentes tejidos pueden ser las responsables de la asociación que existe entre la nutrición en edades tempranas y su influencia en las funciones fisiológicas a largo plazo (RAZIN 1998). La metilación del DNA o la acetilación de histonas son susceptibles de producirse bajo influencia nutricional, como se ha demostrado en estudios animales cuya dieta ha sido suplementada con ácido fólico, vitamina B12, colina o betaína (WATERLAND and JIRTLE 2003).
- **Expresión génica:** La expresión de genes en los diferentes tejidos puede también verse modificada por la dieta de la madre. Por ejemplo, en la glándula mamaria, las intervenciones dietéticas pueden producir modificaciones a este nivel, de igual modo pueden ocurrir también en otros tejidos como el hipotálamo, el tejido adiposo o el útero.

Aún no se sabe con exactitud la relación entre los cambios génicos y la remodelación del tejido, los cambios epigenéticos y el papel de otras estructuras. La situación es extremadamente compleja y se requieren estudios detallados para conocer el mecanismo exacto que promueve dichos cambios en tejidos determinantes (LANGLEY-EVANS 2006).

3.1 PROGRAMACIÓN TEMPRANA EN LA OBESIDAD

La obesidad infantil es considerada una enfermedad de orden epidémico, y tiene consecuencias a corto y largo término en el rendimiento, calidad de vida, salud y esperanza de vida. Por la dimensión del problema es necesario disponer de mecanismos que traten la obesidad infantil, pero hasta el momento no han dado grandes resultados (SUMMERBELL *et al.* 2005). Por lo tanto, en este contexto, las estrategias deben ir dirigidas hacia el desarrollo, evaluación e implementación de medidas preventivas de la obesidad. Existen un gran número de estudios que avalan el hecho de que modificar la nutrición infantil, por ejemplo promoviendo la lactancia materna, puede contribuir a la disminución del riesgo a padecer obesidad en edad adulta (SCHACK-NIELSEN and MICHAELSEN 2006). Como ya se ha mencionado, se han publicado estudios en diferentes condiciones que confirman la protección que procura la leche materna sobre el riesgo de padecer obesidad y sobrepeso (LEE and LORENZ 1978; DE MOURA and PASSOS 2005; HERRERA *et al.* 2006; HADSELL *et al.* 2007; PALOU and PICÓ 2009; PICO *et al.* 2011; SERVERA *et al.* 2012). Por otro lado, la ingesta elevada de proteínas (significativa en algunas leches de fórmula), pero no de energía, grasas, o carbohidratos ha sido también relacionada con un aumento en la adiposidad en edades tempranas y un consecuente mayor IMC en edad más avanzada (PARIZKOVA and ROLLAND-CACHERA 1997). Sin embargo, si durante la gestación se alimenta a la madre con dietas bajas en proteína, también se produce un desequilibrio en el balance energético que luego conduce también a la obesidad; el mecanismo subyacente es una alteración en las preferencias alimentarias: las crías de los grupos alimentados con la dieta hipoprotéica muestran mayor inclinación hacia los alimentos ricos en grasa (BELLINGER *et al.* 2004; LANGLEY-EVANS 2006).

Por otra parte, la obesidad y otras patologías metabólicas están influenciadas por el ambiente nutricional de la madre en la etapa perinatal de modo que la comprensión de los cambios genéticos y epigenéticos producidos y su relación con el desarrollo de obesidad abre la posibilidad de realizar intervenciones específicas y fundamentadas para mejorar esa predisposición genética (LILLYCROP and BURDGE 2011).

La obesidad es una enfermedad crónica cuya característica principal es un aumento de las reservas energéticas del organismo en forma de grasa en el tejido adiposo que repercute en un aumento de peso del individuo. Existen diferentes depósitos de tejido adiposo y su caracterización funcional proporciona información sobre las diferentes adaptaciones metabólicas y su dependencia asociada a intervenciones específicas durante la lactancia

(HERRERA *et al.* 2005). El tejido adiposo se puede diferenciar en tejido adiposo *blanco* y *marrón*. Ambos presentan características morfológicas diferencias, pero también difieren en su distribución, expresión génica y función. Los adipocitos del tejido adiposo marrón son multiloculares y ricos en mitocondrias capaces de expresar cantidades significativas de la proteína desacoplante 1 (UCP1), responsable de la actividad termogénica de este tejido; mientras que los adipocitos blancos son uniloculares y participan en la regulación del balance energético gracias a la producción de leptina, hormona que informa al cerebro del estado nutricional del individuo para regular la ingesta y el gasto energético (CINTI 2005). La principal función del tejido adiposo es, por tanto, el almacenamiento de energía en forma de triacilglicéridos (TAG), aunque hoy ya se considera evidente que tiene otras funciones fisiológicas importantes, secretando proteínas que participan en la regulación autocrina y paracrina dentro del propio tejido y en la modulación de la función de órganos distales, como el músculo, el páncreas, el hígado o el cerebro. A estas proteínas se les denomina bajo el término común de adipocitoquinas y están implicadas en la regulación del peso corporal (leptina, adiponectina), la función del sistema inmune (TNFα, IL-1 e IL-6), la función vascular (angiotensina, inhibidor del plasminógeno tipo I), la función reproductiva (estrógenos) o el desarrollo de resistencia a insulina (resistina). Por lo tanto el tejido adiposo es un órgano endocrino mayor, lo que permite entender la relación entre el exceso de grasa y los estados patológicos asociados.

Aunque el tejido adiposo blanco y el tejido adiposo marrón están generalmente localizados áreas anatómicas diferenciadas, recientemente se han descrito depósitos de células parecidas a adipocitos marrones (que expresan UCP1) infiltradas en tejido considerado adiposo blanco, incluso en humanos en edad adulta (NEDERGAARD *et al.* 2007; SAITO 2013). Bajo ciertas condiciones como la exposición al frío, la activación del receptor β-adrenérgico, ciertos fármacos o agentes nutricionales, estos adipocitos del tejido adiposo blanco pero que comparten características con los adipocitos marrones se activan y pasan a tener un cierto potencial termogenético. Recientemente se ha profundizado en el estudio de estas células, que también se denominan "brite" (del inglés *brown-in-white*), por estar entre el tejido adiposo marrón y el blanco. El interés de las células "brite" reside en su capacidad potencial de actuar quemando la grasa almacenada en el tejido adiposo blanco y por tanto en su utilización terapéutica en el control de la obesidad (PETROVIC *et al.* 2010; GBURCIK *et al.* 2012; WALDÉN *et al.* 2012; BONET *et al.* 2013). Al menos teóricamente, existen varias vías de actuación. Por un parte, se ha postulado que los adipocitos "brite" podrían provenir de un linaje celular

independiente al de los adipocitos blancos, de modo que la activación de su proliferación e inducción de su programa termogenético sería un punto de actuación estratégico en la lucha contra la obesidad. Por otra parte, también se considera posible la transdiferenciación de adipocitos blancos en "brite", lo cual posibilitaría la eliminación del excedente de grasa. En definitiva, el conocimiento del proceso molecular que dirige la diferenciación y proliferación de adipocitos "brite" es de gran interés en estos momentos en la lucha contra la obesidad y enfermedades metabólicas asociadas.

Las células "brite" expresan un set de genes, caracterizados en estudios transcriptómicos, que pueden definirse como biomarcadores de la presencia y/o actividad de estas células en la masa adiposa donde se localizan. En roedores, el tejido adiposo más apropiado para el estudio de estas células es el inguinal, ya que es donde se ha encontrado mayor capacidad de inducción de adipocitos "brite". Aunque este es un área de investigación muy reciente y dinámica, se han definido una serie de genes cuya expresión se puede asociar a la presencia y características de células "brite"; entre ellos se encuentran: CIDEA (del inglés, Cell death-inducing DFFA-like effector, Hoxc9 (homeobox C9), Shox2 (del inglés, stature homeobox 2), Prdm16 (del inglés, PR domain containing 16) y TBX15 (T-box 15).

Un aspecto de interés que no ha sido abordado es la caracterización del potencial "brite" en individuos de diferente *sexo*. La programación metabólica en la gestación y la lactancia, produce adaptaciones diferenciadas entre los machos y hembras de la progenie. Existen evidencias de que la expresión génica y los cambios epigenéticos de la descendencia son género dependientes y la influencia de los cambios dietéticos que se dan en las madres, afectan de manera diferente a su progenie en función del género. Por ejemplo, la restricción calórica durante la lactancia mejora la sensibilidad a insulina y leptina de las crías, pero es dependiente del género, como se observa en los niveles de mRNA expresados en tejidos como el hipotálamo o el tejido adiposo (PICÓ *et al.* 2012); o una dieta hipoprotéica durante la gestación ejerce diferentes efectos, también epigenéticos, sobre el receptor GLUT 4 en el músculo esquelético, según el sexo (ZHENG *et al.* 2012).

En lo que concierne al tejido adiposo blanco, se van vislumbrando los mecanismos que determinan las diferencias entre machos y hembras en su metabolismo, pero aún se sabe muy poco de lo que ocurre en el tejido adiposo marrón. El hecho de que el tejido adiposo marrón se considerara sólo propio del neonato ha tenido como consecuencia que no fuera objeto de

estudio al tratar la adiposidad en humanos. El papel exacto que tiene este tejido en la regulación de energía en los humanos permanece sin esclarecer pero nos parece relevante considerar las diferencias asociadas al género, ya que una mejor comprensión de su regulación permitiría abordar estrategias fundamentadas y más eficaces en el control de la obesidad.

II. Objetivos

El objetivo de esta tesis ha sido estudiar la influencia de la lactosa y la leucina durante la lactancia sobre la descendencia. Este objetivo se enmarca dentro de una de las principales líneas de investigación de nuestro grupo: el estudio de los efectos de nutrientes específicos durante la lactancia en el balance energético, el metabolismo y la composición corporal y su potencial implicación en el desarrollo de obesidad y patologías asociadas en edad adulta. En esta tesis se ha querido precisar la importancia de la leche materna sobre la salud de la progenie y se ha trabajado en una actualización sobre el tema, desde la nueva vertiente que permite la nutrigenómica y las tecnologías –ómicas asociadas (SERVERA *et al.* 2012). Por otra parte, se ha puesto énfasis en dos nutrientes de relevancia en la leche: la lactosa, glúcido mayoritario y al que determinados individuos sensibles pueden desarrollar intolerancia, y la leucina, uno de los aminoácidos esenciales de cadena ramificada, elemento fundamental a lo largo de la vida. La hipótesis central de desarrollo de dicho tema es que la nutrición, particularmente en etapas tempranas del desarrollo, modula y/o condiciona la susceptibilidad a la obesidad en edad adulta.

Partiendo de este planteamiento se ha propuesto:

1) Desarrollar un método de transfección *in vivo* en la glándula mamaria de rata que conlleve a la sobre-expresión del gen de la β-galactosidasa y posibilite la producción de una leche baja en lactosa *in situ*, que permita:

   a. Caracterizar los efectos de la transfección *per se* sobre la madre, en cuanto a la ingesta, la evolución del peso, la grasa corporal, parámetros séricos de glucosa y leptina, y la composición de la leche (manuscrito II).
   b. Determinar el impacto del consumo durante la lactancia de leche reducida en lactosa, con especial atención a sus efectos en la progenie adulta, sobre el peso, la ingesta, la composición corporal y la tolerancia a la glucosa (manuscrito II).

2) Identificar la influencia de la suplementación de la dieta materna con el aminoácido esencial L-leucina durante la lactancia, centrándose en:

   a. La caracterización transcriptómica, a nivel de la expresión génica de genes clave en el metabolismo de la glándula mamaria de las madres suplementadas (manuscrito III).
   b. El análisis de marcadores "brite" y genes clave en las diferentes rutas metabólicas en tejido adiposo, estudiando sinérgicamente a la influencia de la

leucina durante la lactancia, las adaptaciones metabólicas específicas del sexo en la progenie, así como su impacto asociado a una dieta de hiperlipídica en edad adulta (manuscrito IV).

III. Planteamiento experimental

En este apartado se describe el planteamiento experimental desarrollado en la presente tesis así como los diferentes estudios y determinaciones asociados a ello. Los detalles de cada técnica individual utilizada se encuentran especificados en el apartado de Materiales y Métodos y también se describen en los manuscritos correspondientes.

En todos los estudios *in vivo* se han utilizado ratas Wistar como animal de experimentación, suministradas por Charles River (Barcelona). Durante el período de experimentación los animales se han mantenido estabulados a una temperatura de 22ºC y un ciclo de 12h de luz y 12h de oscuridad, con libre acceso al agua y a su dieta correspondiente.

3.1 DISEÑO Y APLICACIÓN DE LA TRANSFECCIÓN *IN VIVO*

En el contexto del objetivo 1, se ha desarrollado y llevado a cabo una transfección *in vivo* de la glándula mamaria en ratas gestantes para inducir la síntesis de β-galactosidasa y así obtener una leche de contenido reducido en lactosa. Este proceso se ha llevado a cabo cumplimentando secuencialmente las siguientes etapas:

1. **Obtención del material genético:** Se ha optado por la elección de un plásmido adecuado que codifique para el gen de la β-galactosidasa en células de mamíferos. En este caso se ha utilizado el plásmido comercial *pTarget*^{TM} (A1410, Promega, Madison) que contiene una versión modificada de la secuencia que codifica para el α-péptido de la β-galactosidasa, con una región promotora/activadora derivada del citomegalovirus humano que permite la expresión de dicho plásmido en células de mamífero de forma constitutiva.
2. **Transformación en bacterias:** Para disponer del plásmido en las cantidades necesarias, es necesario transformar las bacterias con el plásmido de interés y cultivarlas en condiciones adecuadas. La transformación es una técnica de enorme utilidad que permite introducir cualquier plásmido en casi cualquier tipo de bacteria. Para detectar la transformación, el DNA introducido lleva un marcador seleccionable en un medio específico. En este caso se han transformado bacterias *E. coli* de la cepa JM109 (Promega, Madison, WI) con el plásmido codificante para la β-galactosidasa, se han cultivado en un medio selectivo que contenía X-gal, ampicilina e IPTG (un inductor de la β-galactosidasa) y se han seleccionado las colonias que contienen el

gen lacZ, identificables por su color azul (corresponden a las células transformadas con el vector que lleva el gen de la β-galactosidasa funcional, hidroliza el X-gal y se oxida dando un compuesto azul insoluble).

3. **Cultivo de bacterias:** Los clones seleccionados se cultivan entonces durante 24 horas en medio líquido para favorecer su crecimiento en grandes cantidades y en presencia de ampicilina. La ampicilina permite el crecimiento selectivo de las bacterias que han insertado el plásmido, ya que éste les confiere resistencia a la misma.
4. **Aislamiento y purificación del plásmido:** Se ha realizado una lisis alcalina de las bacterias y el atrapamiento del ADN plasmídico en una columna de intercambio iónico *Plasmid Maxi Kit* Quiagen (Madrid, Spain). Tras su elución, precipitación y posterior resuspensión se ha obtenido el plásmido purificado, y se ha determinado su concentración por espectrofotometría (*NanoDrop® Spectrophotometer ND-1000*, NanoDrop Technologies, Wilmington, DE, USA). Una alícuota de este plásmido se ha enviado a secuenciar como medida de control de calidad externa del proceso. Para preparar la solución de transfección el plásmido purificado se diluye en PBS y se acompleja con una solución de DEAE-dextrano en proporción 1/1.39. El DNA cargado negativamente forma un complejo con el polímero de DEAE-dextrano y así, por endocitosis puede penetrar en las células animales. Esta solución de DNA plasmídico ha sido la utilizada en la inyección de la glándula mamaria.
5. **Transfección *in vivo* del gen β-gal:** La transfección *in vivo* se ha realizado entre 2 y 4 días antes del parto en ratas gestantes, alimentadas con pienso estándar (A04, Panlab, Barcelona) y estabuladas en condiciones normales. Se ha llevado a cabo una inyección subcutánea en el tejido mamario de 50 μl de la solución con el plásmido, en cada una de las 12 glándulas mamarias y en condiciones de anestesia con éter. La solución ha sido atemperada antes de la inyección con el objeto de mantener la misma temperatura corporal. Los animales se han dividido en dos grupos: el grupo tratado (n=6), al que se le ha inyectado el plásmido; y el grupo control (n=6), al que paralelamente se le ha inoculado el mismo volumen de la solución anterior sin DNA plasmídico, tan sólo PBS y DEAE-dextrano, con el fin de aplicar la misma manipulación.

3.1.1 Caracterización de las madres

Para caracterizar los efectos de la transfección *per se* sobre las madres, se ha realizado un seguimiento de los animales durante toda la lactancia. El día del parto se fijó como día 0 de

lactancia. El peso, la ingesta, y la composición corporal se han registrado periódicamente hasta día 21. También se han recogido muestras de sangre y leche durante la lactancia (día 6, 12 y 18) para analizar posteriormente los niveles de leptina, glucosa, galactosa y lactosa.

3.1.2 Determinación del impacto en la descendencia

Para estudiar el efecto de la transfección del gen β-gal en glándula mamaria sobre las crías se ha realizado un seguimiento periódico del peso durante la lactancia y también se ha medido la composición corporal a día 10. Además, se han recogido muestras de sangre para analizar los niveles de glucosa y leptina (día 6, 12 y 18). El día del destete se sacrificaron una cría macho y una hembra elegidas al azar de cada madre, se recogieron los tejidos y pesaron para determinar los cambios en la masa tisular asociados al tratamiento. El resto de animales fueron separados por sexos monitorizados hasta la edad adulta (6 meses) midiendo el peso, la ingesta y la composición corporal. Además a los 5 meses se realizó un test de tolerancia a la glucosa para analizar la homeostasis de la glucosa.

3.2 ESTUDIO DE LA INFLUENCIA DE LA LEUCINA DURANTE LA LACTANCIA

En el contexto del objetivo 2, para estudiar el efecto de la suplementación de la dieta materna con leucina durante la lactancia se ha planteado el análisis a nivel transcriptómico la caracterización del metabolismo de la glándula mamaria de las madres, y el del potencial brite del tejido adiposo inguinal de las crías en edad adulta.

3.2.1 Caracterización del metabolismo en la glándula mamaria

Las ratas gestantes han sido alimentadas con dieta estándar en polvo (A04, Panlab, Barcelona). El día del parto se fijó como día 0 y se definieron dos grupos experimentales: C y Leu. El grupo control (n=6) ha sido alimentado con la misma dieta estándar y el grupo tratado (n=6) con dicha dieta suplementada con L-leucina (al 2%) durante toda la lactancia. La L-leucina (>99% NT, Sigma-Aldrich, Madrid-Spain) se añadió directamente al pienso en polvo. La dieta en polvo estándar contiene 1,11 g de L-leucina por 100 g de pienso, por lo que las madres tratadas han recibido un total de 3,11 g de L-leucina por cada 100g de pienso ingerido. Se ha seguido el mismo protocolo y tratamiento descrito previamente (LÓPEZ *et al.* 2010)

Se sacrificaron las madres el día del destete (día 21 de lactancia) por decapitación y bajo condiciones de alimentación al inicio del ciclo de luz. Se procedió a la recogida de la glándula mamaria, que fue pesada y lavada con suero salino que contenía un 0,1% de dietil pirocarbonato que permite su correcta conservación para el análisis transcriptómico. Se guardó a -70ºC hasta su posterior análisis. Una vez extraído el ARN se ha analizado por RT-PCR la expresión de genes relacionados con el metabolismo lipídico (FABP3, FABP4, CAV1, Slc27a1, Slc27a3, SCD1, SCD2, Acly, ATGL, HSL), el transporte de glucosa (GLUT1 y GLUT4), el de aminoácidos (ASC, LAT1, CAT1), la síntesis de lactosa (L-alba) y de proteínas lácteas (CSN1S1, CSN1S2A, CSN2, CSN3), el metabolismo en la ruta de las pentosas fosfato (G6PDH), el ciclo de Krebs (PC, CS, Slc25a1), la insulina (InSR, IRS1, IRS2), los ritmos circadianos (CLOCK), el estrés del retículo endoplasmático (Xbp1, Xbp1s), el balance energético (LEP, UCP2, ADPN) y otros genes asociados con la regulación metabólica (SERBP1c, C/EBPa, HK, STAT3, STAT5 y TGFb). También se han analizado marcadores células brite (CIDEA, Hoxc9, FGF21, TBX15). Adicionalmente se ha procedido a determinar la concentración de lactosa en muestras de leche de día 18.

3.2.2 Análisis de la expresión génica en el tejido adiposo inguinal de las crías en edad adulta

A partir del destete (día 21) todas las crías (procedentes de las madres C y Leu) han sido alimentadas con dieta estándar en cartucho (A04, Panlab, Barcelona). Para facilitar la transición de la dieta en polvo a cartucho, se mantuvieron durante 20 días con ambas dietas. Con el fin de estudiar el impacto asociado a una dieta hiperlipídica, a los 6 meses de edad cada grupo experimental (C y Leu) ha sido subdividido a su vez en otros dos. Uno de ellos ha sido alimentado con una dieta de elevado contenido en carbohidratos (HC), que aporta 3,8 kcal/g y contiene un 10% de las calorías en forma de grasa (D12450[1]). El otro subgrupo ha sido alimentado con una dieta de elevado contenido en grasa (HF) que aporta 4,7 kcal/g y contiene un 45% de las calorías en forma de grasa (D12451[2]). Ambas dietas han sido adquiridas a la casa comercial Research Diets (New Brunswick, USA). A los 9 meses de edad los animales han sido sacrificados en condiciones de alimentación por decapitación y se ha extraído el tejido adiposo inguinal y se han procesado las muestras de forma similar a como se ha descrito previamente (apartado 3.2.1).

Se han determinado los niveles de expresión de determinados marcadores de tejido adiposo marrón o "brite" (PRDM 16, FGF21, UCP1, CIDEA, TBX15, HOXC9 SHOX2) y de genes clave en el metabolismo del tejido adiposo: proteínas reguladoras (LEP, ADPN, RETN), genes implicados en la utilización de ácidos grasos y lípidos (ATGL, LPL), su procesamiento (FASN, CPT1m, SCD2), factores de transcripción (SREBP y PPARγ2), transportadores de glucosa (GLUT4), marcadores de infiltración de macrófagos (CD36), estrés del retículo endoplásmico (XBP) y la proteínas desacoplante (UCP2).

1. http://www.researchdiets.com/system/resources/
2. http://www.researchdiets.com/system/resources/

IV. Resultados y discusión

MANUSCRITO I

Nutrigenomics and Breast Milk, Perspectives in Obesity Lactation: Natural Processes, Physiological Responses and Role in Maternity.

Servera M, López N, Zamanillo R, Picó C, Palou A, Serra F.

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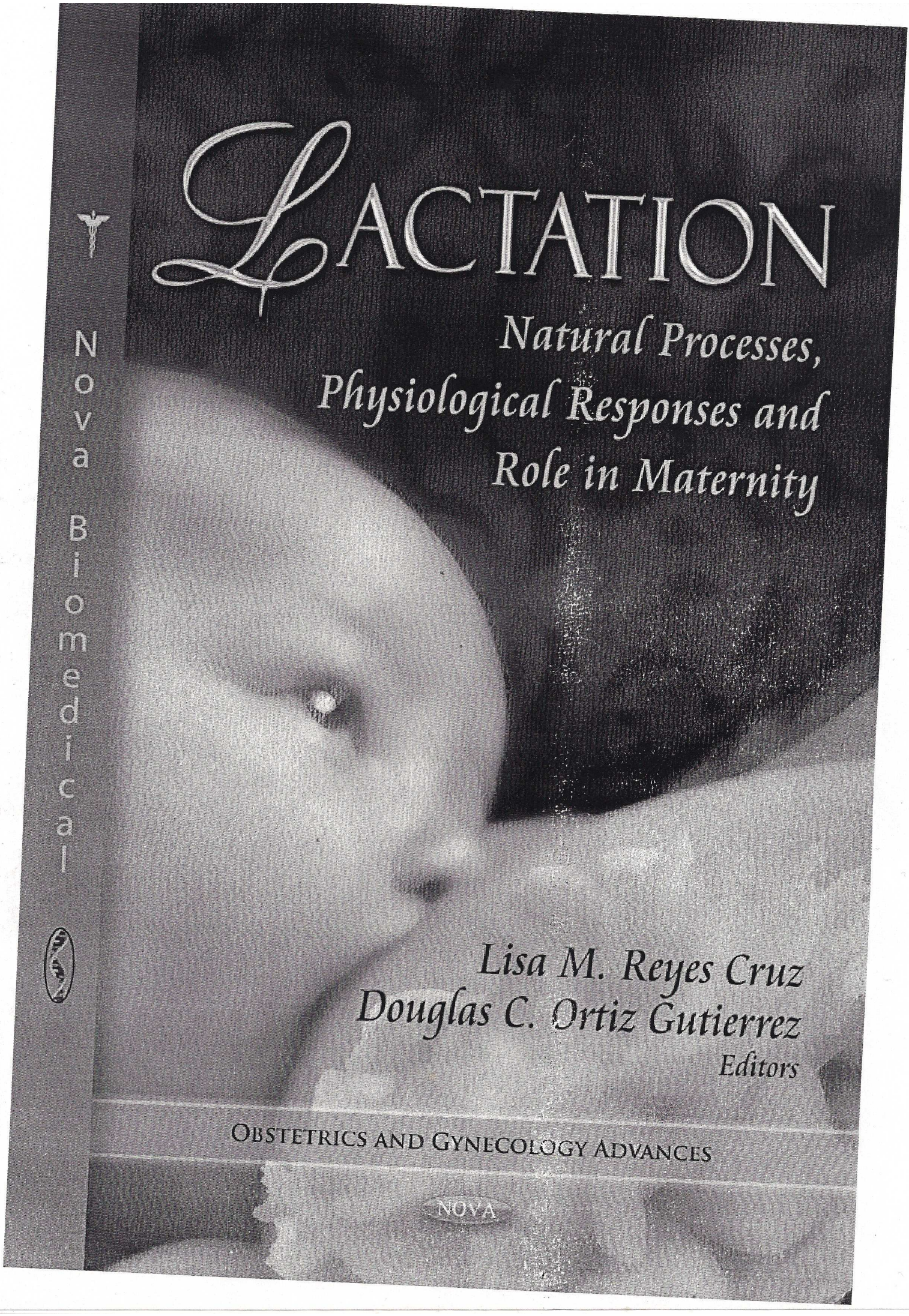
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OBSTETRICS AND GYNECOLOGY ADVANCES

LACTATION

NATURAL PROCESSES, PHYSIOLOGICAL RESPONSES AND ROLE IN MATERNITY

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Contents

Preface

The primary function of the mammary gland is to provide nutrition for the young in the form of milk protein and fat. However, there are other benefits that are provided by lactation, such as the provision of immune factors that are secreted into the milk, which provide protection from infection. In this book, the authors discuss the natural processes, physiological response and role in maternity of lactation. Topics include gene expression in the mammary gland; human milk oligosaccharides and their health effects; the role of micro-RNAs in the lactation process; hormonal control of lactation, the role of breastfeeding in lactation; and Wnt signaling and ultrasound in lactation.

Chapter I - Nutrigenomics is the study of the interaction of dietary factors with genes, examining the influence that certain nutrients have on their expression and therefore may either contribute to health (along with other environmental factors), for example through a healthy balanced diet, or determine the onset of certain diseases (eg obesity). Nutrients modulate molecular processes involving DNA structure, gene expression and metabolism, and these in turn may affect the organism's development. In this context, breast milk, which constitutes the first food, has an essential role not only in promoting children's healthy growth, but also in preventing adult diseases, such as obesity.

This review focuses on the potential modulation of genes by dietary food compounds, devoting particular attention to gene expression in the mammary gland. In addition the knowledge on milk composition from a nutrigenomics perspective and the likely health-outcomes will be analysed, in particular those associated with energy metabolism and body weight control that could be related to changes in milk composition. Finally, the role of milk leptin as a key factor in epigenetics and developmental plasticity is evaluated. Leptin would represent an essential nutrient during lactation in the protection against obesity and its metabolic-related disorders in later life.

Chapter II - Human milk contains high concentrations of all the nutrients required for infant growth and development. The essential proteins, fats, lactose, vitamins and minerals are present in milk in amounts optimal for the growth and development of the neonate. Additionally, human milk contains a broad range of nonessential components with clear health benefits for the newborn. The most remarkable beneficial components in human milk are free oligosaccharides, which comprise the third most abundant component. This abundance is extraordinary because these molecules are not digestible by the newborn's gastrointestinal system, yet they have been conserved and amplified in human milk during evolution. Oligosaccharides in milk actively influence the bacterial species that colonize the

infant's gut. These molecules support the growth of selected bifidobacteria within the intestine, thus achieving a protective microbiota dominated by beneficial bacteria (*prebiotic activity*). Milk oligosaccharides also possess anti-infective and anti-toxic activities as they inhibit pathogens from binding to intestinal cells. This inhibition occurs because some human milk oligosaccharides have functional residues in common with the glycan structures found on the gut mucosa. These glycan structures regulate cell-to-cell communication, and they are also used by enteric pathogens to bind to the mucosa and cause infection. Another therapeutic activity of sialylated oligosaccharides is their contribution to the cognitive development of the infant, as sialic acid is an essential nutrient for brain development and is used for sialydation of brain gangliosides. Since human milk oligosaccharides show extreme diversity in structure and may contain various functional groups, they also exert different bioactivities. The bio-therapeutic properties of human milk oligosaccharides, in particular those of fucosylated and sialylated oligosaccharides, are discussed as well as the analytical methods used to determine their composition and identify the potential for their commercial translation.

Chapter III - Micro-RNAs (miRNAs) are small RNA molecules known to participate in important regulatory mechanisms through the targeting of mRNAs by sequence specific interactions, leading to specific inhibition of gene expression. Ongoing studies have revealed the role of miRNAs in the regulation of mammary gland development but a role in lactation is not yet completely clear. Recently, the identification of significant quantities of selective miRNAs in the milk of a number of mammals, together with the recent characterisation of plant food miRNAs in the blood of people, have precipitated an investigation of the potential role of miRNAs in the regulation of the lactation process. This investigation should include both the process of milk production by the mother and the post-partum development of the young. In order to examine the role of milk miRNAs in the lactation process, the Authors propose a comparative framework for the analysis of lactation. The authors review mammalian lactation diversity and animal models of lactation and recent literature on milk miRNA. They also perform comparative and functional analysis of milk miRNAs and, discuss the function of milk miRNAs as informative markers of both lactation status and maternal physiology, as well as information carrying signals facilitating the timely delivery of maternal development signals to the young.

Chapter IV - Lactation is of paramount importance to the survival, development, and growth of mammalian species. The mammary gland development starts during fetal life.The ductal development is particularly associated with puberty, and the alveolar development is associated with proliferative activity in the luteal phase of the reproductive cycle and in the early stages of pregnancy that leads to formation of the milk secreting unit. Parturition and lactation are two processes that are closely coordinated. Profound changes in several key hormones, such as progesterone, estrogen, prolactin, cortisol, placental lactogen, and insulin, occur early in pregnancy and parturition. These prepare and assure milk production by the mammary gland after delivery. For milk ejection, a neuro-hormonal reflex leading to the contraction of the myoepithelial cells surrounding the alveoli is stimulated by the action the oxytocin. After ceasing the stimulus for lactation, involution by apoptosis leads to regression of the mammary gland to the quiescent non-lactating state. Besides these physiological responses, the hormones can also influence cognitive functions and maternal behavior. Lactation is also important for establishing an affective linkage between mother and baby. This review will focus on natural processes of mammary gland development, on the hormonal control of lactation and on the role of breastfeeding in maternity.

Chapter V - The mammary gland is unique in that most of its development and dynamic morphogenesis occurs postnatally in response to changes in the hormonal milieu. Multiple components of the Wnt/β-catenin signaling pathway have been implicated in mouse mammary gland development. It is clear that the Wnt proteins themselves are important regulators of numerous stages throughout postnatal mammary gland development. Mouse models have been developed to analyze whether β-catenin stabilization, expression of pathway components, or expression of Wnt/β-catenin target genes is sufficient to disrupt mammary gland development and lactation. In addition to the role of β-catenin in the Wnt signaling pathway, it is also a major component of the adherens junctions and as such, has a role in maintaining epithelial integrity, which is essential for lactation. Multiple components of the Wnt pathway are known to have cross-regulation with other signaling pathways involved in the lactogenic phenotype. Furthermore, investigation into the regulation of Wnt pathway components has demonstrated that hormones, such as progesterone, can regulate components of the Wnt pathway. Through various mouse models of mammary gland development, it has become clear that the Wnt/β-catenin signaling pathway is a critical regulator of normal mammary gland development. Interestingly, the specific roles of Wnt pathway regulators and components in lactation have given us insight to alterations that occur in breast tumor development.

Chapter VI - Breast milk is the 'gold standard' of infant nutrition providing not only nutrition for optimal growth but immune protection as well. Many women initiate breastfeeding however few continue to breastfeed for the recommended 6 months (WHO). Management of lactating women is predominately experience-based therefore lack of diagnostic tests and evidence-based treatment is likely to contribute to early weaning. Ultrasound imaging is not routinely used as a diagnostic tool during lactation however new research suggests that is a promising modality capable of identifying both breast and infant sucking pathologies. Imaging of the non-lactating breast is well established however little imaging is performed during lactation.

Ultrasound during lactation is relatively simple provided settings are optimized to accommodate the increased amount of glandular tissue. Furthermore an understanding of the growth of the breast during pregnancy and changes during lactation as well as lactation pathology enhance diagnoses. Ultrasound can also be utilized to confirm normal function of the lactating breast. While sufficient milk must be synthesised for the optimal growth of the infant it must also be released during breast feeding or breast expression by the milk ejection reflex. Increasing duct diameter and visualisation of milk flow at milk ejection confirms that the reflex is intact. A successful lactation depends upon the infant's ability to remove milk from the breast. Infant tongue action can be visualised during both breast and bottle feeding. Recently this technique has been employed to assess infants with oral anomalies such as ankyloglossia. It can also be applied to the infants of mother experiencing pain during breastfeeding to determine if compression of the nipple is a contributing factor. Ultrasound techniques have also been developed to image swallowing in both breast and bottle fed infants but have not yet been used extensively to identify swallowing pathology.

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Chapter I

Nutrigenomics and Breast Milk, Perspectives in Obesity

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Abstract

Nutrigenomics is the study of the interaction of dietary factors with genes, examining the influence that certain nutrients have on their expression and therefore may either contribute to health (along with other environmental factors), for example through a healthy balanced diet, or determine the onset of certain diseases (eg obesity). Nutrients modulate molecular processes involving DNA structure, gene expression and metabolism, and these in turn may affect the organism's development. In this context, breast milk, which constitutes our first food, has an essential role not only in promoting children's healthy growth, but also in preventing adult diseases, such as obesity.

This review focuses on the potential modulation of genes by dietary food compounds, devoting particular attention to gene expression in the mammary gland. In addition the knowledge on milk composition from a nutrigenomics perspective and the likely health-outcomes will be analysed, in particular those associated with energy metabolism and body weight control that could be related to changes in milk composition. Finally, the role of milk leptin as a key factor in epigenetics and developmental plasticity is evaluated. Leptin would represent an essential nutrient during lactation in the protection against obesity and its metabolic-related disorders in later life.

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Mammary Gland

Lactation is a physiological process conditioning offspring growth. Breast milk is the most complete food for the growing infant; it contains all nutrients and substances needed for successful developmental progression of the newborn. As will be shown throughout the chapter, correct feeding during the first months of life has a relevant influence in the future health of the offspring.

Mammary gland has a fundamental role in production and secretion of milk for progeny. Mammary gland morphogenesis begins during embryonic development and continues during the postnatal period. During three major developmental stages—puberty, pregnancy and involution— the gland undergoes profound morphological and functional changes. These changes correspond to periods of cell proliferation, apoptosis and differentiation, in conjunction with changes in gene expression patterns [1-5]. Although mammary gland development enables lactation to occur after delivery, previous environmental inputs along life may condition its development and therefore, the so called epigenetic modulation, may affect the performance of the gland as a synthesizing organ [6, 7].

Epigenetic Evidences in Mammary Gland

Epigenetics is a modulatory metabolic process, characterized by stable alterations in gene expression that arise during specific developmental stages (to be adapted to novel and changing environmental conditions) and may have phenotypical consequences, even in the descendants. Epigenetics is relevant in the context of this chapter because epigenetic dysregulation can cause obesity. Dietary components and other factors, including maternal obesity during pregnancy and lactation, might cause metabolic imprinting in the offspring, perpetuating, or even amplifying, obesity susceptibility across generations. Such epigenetic modifications can affect different organs, tissues and metabolic pathways associated with obesity (adipose metabolism, neural networks of energy balance regulation and so on) [8, 9].

In this section we are going to review the actual knowledge on the potential epigenetic imprinting that may affect mammary gland development and therefore milk composition. Following sections will cover specific knowledge on epigenetic mechanisms affecting milk composition and, if it is known, the impact on body weight regulation of the newborn.

At a biochemical level, epigenetic changes lead to alterations in chromatin conformation. These changes in chromatin are brought about by DNA methylation, histone modification, non-histone chromatin proteins and even non-coding RNAs (ncRNA) [10-14].

The most characterized epigenetic mechanism is methylation. Methylated cytosines serve as docking sites for proteins that prevent transcription factors from accessing their binding sites on the gene promoter. Histones are also susceptible to modification by a number of functional groups, including methylation at specific amino acids and, as a consequence, chromatin accessibility to regulating factors is amended and adapted to environmental stimulus. Extensive work in the agouti viable yellow (A^{vy}) mouse provided the first clear demonstration that supplementation of the mother's diet during the perinatal period, with nutrients affecting availability of compounds involved in the methylation pathway, can cause a permanent phenotypic change in the offspring via an epigenetic mechanism. Specifically,

genotypic obese mice show a lean phenotype associated with hypermethylation of the agouti locus. Recent studies provide compelling evidence that epigenetic regulation at specific genomic loci is susceptible to environmental influences during development in humans [8, 15].

Some studies have reported the inverse correlation between expression of major milk protein genes and methylation status, of either the promoter or the regulatory regions, in the lactating mammary gland, which occurs in pregnancy and puberty. This is partly modulated by lactogenic hormones, which act inducing an open chromatin conformation at regulatory regions. Evidences of other epigenetic mechanisms associated with transcription activation of mammary gland during lactation have also been documented [6].

Gene Expression in Lactating Mammary Gland

Physiology, gene expression and regulation in human mammary gland are not well known because of the ethical and practical problems associated to sampling. *In vitro* studies lack the complexity of the number of cells involved in breast: adipocytes, stromal and epithelial tissue, macrophages and lymphocytes. Some existing data on gene expression in human mammary epithelium have been obtained from milk fat globules (MFG). Mammary epithelium cells (MEC) RNA is isolated from MFG and the obtained transcriptome of human MFG is similar to that described in mammary glands of lactating rats [16]. A number of comparative studies have been addressed to analyze gene expression of mammary glands from different animal species in relationship with specific functions (e.g. immune function) [17] and in comparison with human milk composition [2]. Mechanisms that regulate proliferation, apoptosis, differentiation and tissue remodeling of mammary glands have been studied at different stages in different species and compared with other tissues, such as liver or adipose tissue by microarray [18], RT-PCR techniques or immunohistochemistry [19]. Recently, novel bioinformatic tools have been applied to data sets to get some insight on the potential regulatory gene networks in lactation [20]. However, molecular physiology of human lactation is almost unknown and most of the knowledge has been inferred from animal or cellular models [16].

The ability to secrete milk develops during pregnancy when the mammary gland is transformed from a simple ductal tree to a highly efficient exocrine organ with expansive lobular-alveolar structures. There is marked proliferation of ductal and alveolar epithelial cells during pregnancy, implying a progressive reorganization of the mammary gland, replacing adipose tissue by secretory epithelium. Secretory differentiation of the mammary gland begins around mid-pregnancy, but is at parturition when the secretory activation is fully functional and homogeneous expression of milk protein genes and biosynthetic enzymes by alveolar cells is reached [2, 21].

The mammary epithelium is composed by two layers of cells: luminal and basal cells. Luminal cells produce and secrete milk whereas basal or myoepithelial cells are responsible for the contraction that allows milk output. The correct development and function of the mammary gland during lactation depends on genes such as IRS-1 and -2 (Insulin receptor substrate 1 and 2) or IGF-1 (Insulin-like growth factor 1). These genes are sensitive to hormones that regulate postnatal development of the gland and are involved in either

apoptosis or stimulation of mammary epithelial cells. These protein genes, highly induced in mammary gland, are involved in lactogenesis and in milk synthesis [22, 23].

The Lactome

The lactome, comprising the subset of genes responsible for lactation, is spatially and temporally complex [24]. MEC are essentially biofactories of lipids, proteins and carbohydrates for milk during lactation. Milk production goes by hand with a broad suppression of functions to effectively push all of the cell's resources towards the massive synthesis of lipids and of a large quantity of a minority of proteins. However, apart from small subsets of proteins such as the major milk proteins and lipid synthesis, there is no a sudden transcriptional switch around the time of parturition [20].

Gene ontology analysis suggests that much of the machinery for the secretory pathway is transcribed prior to lactation. Therefore, preparation of the mammary gland for lactation includes modifications of the transcriptional program, but the onset of lactation appears to be primarily controlled by post-transcriptional mechanisms [20, 25, 26].

Major milk protein genes: As a group, genes transcribed to synthesize major milk proteins are up-regulated sharply around the time of parturition. However, this represents a small subset of genes and the secretory pathway is likely devoted to the secretion of large amounts of a few proteins. For instance, highly expressed milk protein genes, such as the caseins, account for as much as 30% of total RNA. Concerning post-transcriptional mechanisms commented above, the mRNA of casein genes accumulates rapidly due to increased RNA stability in presence of lactogenic hormones [27]. Enhancement of translation of the mRNA of the β-casein protein occurs by lengthening the poly(A) tract, synergized by the activity of both prolactin and insulin signaling [28]. The rate of translation has also been found to be reduced by amino acid deprivation [29].

Fatty acid metabolism genes: Milk triglycerides (TG) are synthesized from substrates derived from exogenous (dietary) fats and from fatty acids that are synthesized *de novo* in the gland. These pathways converge at the point where short, medium and long chain fatty acids are esterified to a common glycerol backbone. As mentioned, the relative mRNA levels of the main TG synthesis enzymes begin to increase on average at about mid pregnancy and then remain elevated throughout lactation. This has been described for glycerol kinase (GK), which phosphorylates the glycerol imported from the plasma, the long chain acyl-CoA synthase (LACS4), which supplies the mammary epithelium with a pool of acyl-CoA, and for acylglycerol-3-phosphate-acyl transferase (AGPAT), the enzyme which acylates the sn-2 hydroxyl group of the glycerol backbone [26].

The mRNA levels for enzymes providing long chain polyunsaturated fatty acids (LC-PUFA) to the neonate are also significantly upregulated starting at mid pregnancy. These include Elovl-1, that elongates very long chain fatty acids, and a Δ5 fatty acid desaturase (FAD1) [30]. None of these genes respond to dietary lipids, since TG synthesis must remain approximately constant to maintain total milk lipid content. In contrast, mRNA for most enzymes of the β-oxidation pathway falls 2- to 3-fold at secretory activation, to allow all lipid substrates to be diverted towards milk fat synthesis. In addition, fatty acid synthesis is activated, particularly when the dietary supply is not enough to cope with the demand for milk synthesis. This process requires the coordination of multiple genes that control the flux of substrates, including enzymes, transporters, binding proteins and others. As happens in liver, sterol response element binding protein1 (SREBP1) seems to be the transcription factor

responsible for induction of the lipogenic genes in mammary gland such as fatty acid synthase (FASN), the stearoyl-coenzyme A desaturases (SCD1 and SCD2) and the mitochondrial citrate transporter (SLC25a1)[31].

Plasma membrane substrate transporters: The synthesis of lipid and lactose in the mammary gland begins with the transport of precursors or substrates from the plasma. The transport of glucose across the plasma membrane is important for both lipid and lactose biosynthesis. Therefore, a number of membrane transporters are induced to facilitate the transfer of glucose into the mammary gland. Glucose is conveyed into the cells either discretely by the family of GLUT transporters, which are dealing with facilitated transport, or coupled to a sodium-dependent mechanism (Slc5a1) [32-34]. The mammary gland also utilizes fatty acids mobilized from other metabolic tissues, specifically liver and adipose tissue. Fatty acids enter cells through both passive diffusion and by protein-mediated transfer mechanisms facilitated by transporter proteins, including fatty acid translocase (FAT/CD36), fatty acid binding protein (FABP) and caveolin [35-37]. Regarding the amino acids necessary for protein synthesis in the mammary gland, a number of transporter genes are also expressed in mammary gland, including the transporter ASC (specific for alanine, serine and cysteine), LAT1 and SLC3A2, with are specific for neutral amino acids, and the Tau or Slc6a6, specific for taurine. These transporters are not expressed at the same degree throughout time but its expression is able to respond to metabolic needs of the gland and to the protein intake of the mother, and may even be susceptible to hormonal changes [32].

Mineral binding/transport capacity: One of the nutritional properties of milk is the co-delivery of minerals, implying that the processes of lactation must include substantial ion transport capabilities. Gene ontology analysis also suggests that the increased transcription of genes associated with ion transport begins during pregnancy. Both zinc and iron transport systems are up-regulated in lactation (ceruloplasmin, ferritin heavy chain, lactotransferrin, sideroflexin 2 and transferrin receptor). However, some other genes associated with iron transport are also downregulated (ferritin light chain 1, hephaestin, hemochromatosis, sideroflexin 1 and solute carrier family 11 member 1 (Slc11a1)) [32].

Dietary Influence on Gene Expression and in Milk Composition

Environmental conditions, maternal status and dietary inputs may affect either positively or negatively the regulation of gene expression in mammary gland and therefore may affect the composition of breast milk. Subtle variations in the nutritional content and therefore, in milk quality, may not be critical for the success of the offspring but may contribute to perform specific imprinting in lactating neonates leading towards higher propensity to metabolic diseases in adulthood.

It has long been known that large inter-individual variations and changes in composition occur during the course of lactation. This goes together with the fact that breast milk volume and composition from the same individual may vary over the course of the day, during the course of suckling (difference between fore- and hind-milk) and considerably from day to day. In addition, changes in composition are greatest and occur most rapidly during the first week post-partum. The milk produced in the first few days after birth (colostrum) is higher in protein, vitamins A, B12 and K and immunoglobulins than mature breast milk but it is somewhat lower in fat content and hence in energy. Over the following week, the

composition of milk is transitional and, at about 10 days after birth, milk can be considered "mature" (see [38] for a review).

Nutrition during pregnancy directly affects mammary gland development, milk production and percentage of lactose, and the effects on growth of the offspring have been described in domestic animals [39]. In humans, the influence of maternal factors such as diet and body composition on milk composition has been studied and, although the postnatal feeding regime greatly determines infant growth trajectories, there is still a lack of substantial knowledge. For example, weight gain during the first week of life has been shown to be positively associated with later obesity and it is now clear that an earlier age of adiposity rebound is associated with later higher obesity risk [40, 41]. In this sense, the protective effects of breast feeding are documented and a potential role for milk leptin has been proposed [42-44]. However, in general there is a lack of association concerning the potential modulation of macronutrient milk composition by maternal diet, which is consistent with the buffering capacity of the mammary gland against fluctuations in maternal dietary intake or nutritional status [45-48].

In this respect, a number of studies in rats have shown that maternal undernutrition during lactation may influence body weight of lactating offspring, mainly associated with changes in protein and lipid milk concentration. Appropriate supply of protein in maternal diet is necessary to support protein load in milk and progeny growth. In this sense, protein-restricted rats show a deficient development of mammary glands and prolactin secretion, which results in lower protein concentration in milk. This is accompanied by long-lasting effects on liver and muscle mitochondria, and has been associated with the development of insulin resistance in later life. Similarly, energy-restricted dams have higher lipid milk content and, as a result, the offspring is heavier than controls in adulthood [49].

Concerning other important nutrients in milk, such as fatty acids, unbalanced ω6 relative to ω3 LC-PUFA intake in maternal diet has been pointed out as one of the factors contributing to the upward trend in childhood obesity. However, two recent systematic reviews have highlighted the paucity of robust data from both human [50] and animal [51] studies that could contribute to confirm the effect of a maternal diet supplemented with LC-PUFA on body fat mass in the progeny. Docosahexaenoic acid (DHA) is a biologically important fatty acid, present in high concentrations in the brain and retina, and accumulates rapidly in neural tissues during infancy. Therefore, sufficient provision of DHA is thought to be essential for optimal visual and neurologic development during early life. DHA levels in human milk vary considerably, mainly as a consequence of differences in maternal intake of DHA [52, 53] and DHA supplementation of lactating women increases breast milk DHA content [54, 55]. Milk micronutrients constitute the only source of vitamins and oligoelements for the lactating newborn. A general lack of knowledge about the effects of perinatal supplementation with individual minerals/vitamins or multicomplexes and future health outcome in offspring is still lacking [56]. Much attention has been devoted to folic acid because it is essential in the synthesis of DNA, especially during periods of rapid growth and cell division, and has a role in epigenetic mechanisms (see Micronutrients in the following section). Also, lactating women have higher folate requirements due to its draining from breast milk. Folate concentration in human milk is tightly regulated and not affected by maternal folate status except in clinically folate deficient mothers [57]. Something similar occurs with milk zinc (Zn), whose concentration is maintained over a wide range of dietary Zn intake [58-60].

Therefore, future research is needed to establish better evidence of the effects of maternal diet composition during lactation on health in both mother and offspring, particularly for those nutrients whose presence in milk is directly related to the maternal dietary content.

Human Milk Composition

Milk is a very complete food, at least for the first period of the infant's growth. In addition to its well known and characterized content in macro- and micronutrients, breast milk contains hundreds of components, as well as complex macromolecular structures, whose presence has not been fully described and provide benefits in ways we still do not understand, but as outlined above, those may stand out throughout life [24].

It is not the aim of this section to give a detailed description of milk composition, which can be found in many previous reviews. In this section, some of the potential compounds that could play a role in future health outcomes of progeny, particularly concerning modulation of the risk of obesity in adulthood, will be presented. A core of functional components, whose presence in milk is recently beginning to be recognized as beneficial to the newborn and go beyond simple nutritional purposes, will also be analyzed.

Breast-Milk Lipids

The lipid composition of human milk is conditioned by recent maternal dietary fat intake, internal fatty acid composition, fats mobilized from adipose tissue stores and *de novo* lipogenesis during lactation. The contribution differs between sources i.e. about one-third of milk fat linoleic acid (LA) originates from diet whereas the dietary contribution of arachidonic acid (AA) represents only 10%; the other 90% of human milk AA originates from adipose tissue [61].

TG are the main components in milk, but their fatty acid composition and structure is variable. For example, when consuming a low fat diet, specific unsaturated medium chain fatty acids (MCFA) are synthesized in the mammary gland, which are more easily absorbed by the offspring than longer ones because they do not require the carnitine transport process. In addition, human milk is characterized by high palmitic acid content taking part of TG in the specific sn-2 position. This structure is relevant because it affects bioavailability of other nutrients and improves fat and calcium absorption in the infant gut [3, 62, 63].

Milk Fat Globules and Lactosomes

Traditionally, milk lipids have been characterised by their mono-, di- and TG content and their fatty acid-associated composition because these neutral lipid represent around 99% of total milk lipids. However, milk contains a complex mixture of protein-lipid ensembles which have specific synthetic and secretion pathways and probably different nutritional/functional roles. Once lipids are synthesised in the mammary gland, its secretion takes place as lipid droplets of variable size, coated by proteins and polar lipids. According to the droplet volume, at least two different species can be described, MFG and lactosomes, which differ in their lipid and protein composition and appear to be secreted from different pathways. MFGs are

present in cream, which are rich in TG and have the biggest volume (5-10 μm). Lactosomes are found in the skim fraction and are rich in a variety of phospholipid species, almost devoid of TG and cholesterol, and have smaller size (≅25 nm). Thus, it has been suggested that MFG would constitute the main lipid source to provide energy to infant, while lactosomes would potentially offer immunomodulatory functionality independent of macronutrient supply [64-66].

Long Chain Polyunsaturated Fatty Acids

Human milk fat also contains LC-PUFAs. These fatty acids are membrane components that act as second intracellular messengers, modulate gene transcription and serve as precursors for eicosanoids and docosanoids (leukotrienes, prostaglandins, lipoxins and resolvins) synthesis. Because of the limited ability of infants to synthesize them from essential fatty acids, human milk practically constitutes as the unique LC-PUFAs source, and maybe for this reason colostrum has the highest concentration and it decreases with maturation of milk.

Approximately 30% of LC-PUFA milk content derives from the maternal diet, while the major part comes from maternal body stores, thus maternal long-term dietary intake influences milk fat composition [63, 67, 68].

In addition, endogenous synthesis of LC-PUFA in the mammary gland takes place depending on diet and nutritional status, which can be assessed by analysing desaturase and elongase activities in plasma. Obesity and metabolic syndrome have been associated with high Δ9-desaturase and Δ6-desaturase (Fads2) activities and low Δ5-desaturase (Fads1) activity in plasma. Thus, obesity as well as maternal diet during lactation may influence milk fatty acid composition. On the other hand, elongase activity has been recently recognized as another key control point for LC-PUFAs synthesis. Specifically, the presence and the activity of Elovl2 seem to be critical in understanding whether DHA synthesis can be increased by dietary means. In fact, supplementation of lactating women with DHA precursor has little effect on breast milk DHA content [50, 51, 53, 69-71].

Another aspect of concern is the fact that the increased consumption of vegetable oils (rich in ω6 PUFAs) in detriment of seafood intake (ω3 enriched) during the last decades has been associated with increased ω6/ω3 ratio in breast milk. In particular, a significant time-increase of LA content has been systematically reported in breast milk of women living in major industrialized countries of the Western world since the fifties, whereas α-linolenic (LNA) content has not changed in a parallel way, leading to a continuous increase in the LA/LNA ratio. Independently of mammary gland lipid metabolism, this ratio varies between 5:1 and 15:1 in breast milk of women in westernized countries. Such an imbalance of the precursor ratio may favour the conversion of LA to ARA to the detriment of the synthesis of eicosapentaenoic acid (EPA) and DHA from LNA. Consistent with the adipogenic role played by LA and the anti-adipogenic role played by LNA, the ω6/ω3 ratio is involved in the determination of body composition. In animal studies, α-linolenic acid (ALA) or ω3 LC-PUFAs supplementation during early development has been associated to an improvement in body composition of the offspring, being more noticeable in males. In humans, an enhanced maternal ω3 PUFAs status has been associated with lower childhood adiposity too. This obesity modulatory potential has been associated with the ω3 fatty acids potential to decrease serum leptin levels during suckling in offspring, even though milk leptin level is not affected, thus suggesting an endogenous effect on the regulation of its synthesis in offspring. In

addition, a high ω6 intake may favour transformation of inflammatory products (such as eicosanoids or prostaglandins) synthesized from AA that may contribute to the inflammatory status characterizing many metabolic diseases including obesity [50, 72-76].

Furthermore, the impact of increased dietary LA:LNA ratio maintained over generations (in combination with a high-fat diet) triggers a discrete and steady increase in inflammatory stimuli accompanied by enhancement of fat mass, which suggest the involvement of epigenetic mechanisms in transgenerational obesity [77].

Moreover, LC-PUFAs are molecules with the potential to modulate the levels of hypothalamic peptides responsible of the neuro-regulatory circuitry for the feeding behaviour control. In rats who are naturally susceptible to metabolic syndrome, an isoenergetic diet with higher EPA and DHA and lower LA increased hypothalamic expression of the anorexigenic peptide cocaine- and amphetamine-regulated transcript (CART) [78]. Therefore, LC-PUFAs may modify neuronal plasticity and leptin signalling pathway in hypothalamus.

Therefore, a number of evidences have led to the proposal that an imbalance in the ω6/ω3 ratio during critical stages of development may be one of the factors contributing to the upward trend in childhood obesity [50, 51, 79].

Other Fatty Acids

Conjugated linoleic acids: Other kind of human milk PUFA components are conjugated linoleic acids (CLA) and conjugated alpha-linolenic acids (CLNA). CLA is present in breast milk and increases in response to maternal intake [80]. Some of them, and in particular a 50% mixture of the two main isomers (c9,t11-CLA and t10,c12-CLA), exhibit hypolipidemic and antiobesity effects in animals and are used also in the management of human obesity [81-87]. The impact of CLA on body weight or composition of lactating infants has not been assessed.

Saturated fatty acids are often associated to unhealthy outcomes. However, they can also have beneficial roles in the body. Human milk contains butyric (C4:0, 0.4%), caproic (C6:0, 0.1%), caprylic (C8:0, 0.3%), capric (C10:0, 1.2%), lauric (C12:0, 5.8%), myristic (C14:0, 8.6%), palmitic (C16:0, 22.6%) and stearic (C18:0, 7.7%) acids. Butyric acid lowers inflammation processes in the intestine acting through specific receptors; caproic, caprylic, capric and lauric acids act as potent antimicrobiological agents across gastrointestinal tract; myristic acid, especially in the sn-2 position of milk triglycerides, is responsible to rise blood HDL levels and palmitic acid may promote lipoprotein metabolism in infants through peroxisome proliferator-activated receptor gamma coactivator-1 (PGC1) stimulation in the liver. Overall, saturated fatty acids from human milk, some of them being of short chain not usually present in other foods, seem to carry out biological functions of relevance in relation with metabolic disturbances [88].

Milk Sterols

Cholesterol is a major component of the brain, accounting for 2–3% of weight and 20–30% of all lipids in the brain. Human milk provides cholesterol between 10-20mg/dL, which is higher than the usual content in infant's formula. A systematic meta-analysis has shown that total blood cholesterol concentrations in breastfed subjects, compared with those in formula-fed subjects, were higher in infancy, similar in childhood, and lower in adult life. Thus, breastfeeding has been associated with lower blood cholesterol concentrations later in life and could be one of the potential factors contributing to the metabolic programming of healthy adulthood [63, 89-92].

Phytosterols: Breast milk also contains phytosterols (1.7 mg/dL) and their intake is followed by parallel changes in maternal milk and in both maternal and infant plasma [93]. This may be of importance in the context of high maternal intake of phytosterols, either from natural or from enriched food products. Dietary phytosterols are relevant because of their use as cholesterol-lowering agents to reduce incidence of cardiovascular disease (CVD). A recent systematic review and meta-analysis, though, has questioned evidences of association between serum concentrations of plant sterols and risk of CVD [94].

In general, the diversity of molecules involved in the term dietary lipids make them compounds with multiple potential roles in metabolism. They are potential signalling molecules involved in appetite and energy metabolism, metabolic sensors in the regulation of energy storage/oxidation, mediators of the control of gene expression, and so on. Therefore, specific milk lipids may be considered as important factors with the potential to contribute to metabolic programming during lactation.

Milk Proteins

Milk proteins have been studied in depth for decades and knowledge on immunoglobulin abundance in milk has allowed characterizing the protective and immunological role of milk in newborns. Recently, and thanks to available proteomic techniques, a novel set of proteins present in human milk have been found and are under characterization [95, 96].

Traditionally, milk proteins are grouped into three main fractions: caseins (80%) found in micelles in the form of colloidal dispersion; whey proteins found in the soluble fraction and mucine proteins associated with the MFG membrane (16%) and therefore most involved in lipid delivery. Milk protein content and composition is a changing medium that evolves along lactation together with newborn development.

Biological functions of milk proteins are mainly associated with immune response, cell proliferation/differentiation and lipid metabolism/nutrient transport.

Concerning the nutrient transport role, milk proteins provide an important source of amino acids to the rapidly growing breastfed infants, but in addition they may have other complementary roles. Therefore, casein and whey fractions are involved in facilitating the digestion and uptake of other nutrients in breast milk. Some proteins are able to bind nutrients and assist their delivery in the intestinal mucosa; this is the case for β-casein and lactoferrin, which help in the absorption of calcium and iron, respectively. Specific proteins have been found to assist degradation of mono-, di-, and triacylglycerols and cholesterol esters, TG, complex carbohydrates, bioticidin and glutathione. For example, γ-glutamyltransferase is a protein involved in transferring the glutamyl moiety of the milk glutathione to a variety of acceptor molecules, yielding cysteine as an available amino acid essential for redox homeostasis and neonatal growth/development. In addition, various inhibitors of proteases are present in milk, which have been proposed to limit the activity of pancreatic enzymes, reducing protein digestibility and allowing essential polypeptides to reach the intestinal tract. Some of these inhibitors have a dual role in regulating complement activation/inflammatory processes, supporting the cross-talk between nutrient delivery and immune response pathways/interactions. Furthermore, milk proteins are a source of biologically active peptides. A number of milk proteins are resistant against proteolysis in the gastrointestinal tract and contribute, in an intact or partially digested form, to the release of an arsenal of encrypted

bioactive peptides in the gut lumen. This cryptome, still poorly investigated, seems responsible for concretization of number of physiological activities with hormone-like activity [97].

In this review, two specific aspects of milk proteins will be addressed, those which have a latent functionality which arises after enzymatic cleavage in the intestinal tract and those which have a modulatory role, being substances that have the potential to act as mediators between mother and child and contribute to establish biochemical and physiological communication during lactation. The latter aspect may include hormones, growth factors, cytokines and even whole cells.

Bioactive Peptides in Milk Proteins

Milk proteins constitute the main source of bioactive peptides with a functional role in newborn development. Bioactive peptides are generated from milk proteins in the gastrointestinal tract. Due to the relative immaturity of intestinal epithelium in the newborn, peptides may pass through the epithelium and reach peripheral tissues with the potential to act locally or systemically. Bioactive peptides may influence immune system maturation and cognitive development, as well as a possible participation in the prevention of colonization by pathogens, promoting a positive microflora in newborn [24, 98].

The main milk proteins αs1-casein and β-casein have the capacity to liberate about 20.000 peptides each. Once liberated and absorbed, these bioactive peptides may exert physiological effects on the various systems of the body. Examples of milk bioactive peptides include caseinophosphopeptides, which have a role in the transport and absorption of minerals; glycomacropeptides, which bind toxins; casoxins and casomorphins, which act as opioid antagonists and agonists respectively; isracidin, which has immunomodulatory effects and casoplatelin with antithrombotic activity. Other activities identified include angiotensin converting enzyme (ACE) inhibition, antihypertensive and antithrombotic effects, which have been extensively studied as potential dietary tools to be used in the treatment and prevention of cardiovascular disease [99, 100].

However, most of the studies deal with animal sources (fermented dairy products) and their impact in adult humans; whether breast milk has the same potential in newborns and/or conditions future phenotypic features is not known.

Opioid peptides are peptides like natural enkephalins that have affinity for the opiate receptor (as agonists or antagonists) and elicit tissular/cellular opiate-like effects. β-Casein gives way to a number of derived peptides with opioid agonist activity termed β-casomorphins. Other peptides with agonist opioid activity are derived from α-casein (exorphins) or α-lactalbumin (lactorphins). Casoxins (from κ-casein) and lactoferroxins (from lactoferrin) are milk derived peptides with opioid antagonist activity. β-casomorphins are the most characterized concerning their potential activity in newborn gut lumen. Interestingly, no absorption of β-casomorphins in adult intestinal tract has been reported. In contrast, passive transport of β-casomorphins across intestinal mucosal membranes does occur in neonates, which may lead to physiological responses such as an analgesic effect on the nervous system resulting in calmness and sleep in infants. Casomorphins, as opioid ligands, are able to modulate social behaviour, increase analgesic behaviour, prolong gastrointestinal transient time, exert antidiarrheal action, modulate amino acid transport and stimulate endocrine responses such as the secretion of insulin and somatostatin. Opioid-like milk peptides also play a regulatory role regarding appetite by modifying endocrine activity of the pancreas [101].

Modulatory Proteins

Human milk is a rich source of growth factors, several hormones (i.e. insulin and growth hormone) and neuropeptides (i.e. neuropeptide Y) with known functions in the cellular context. However, their presence in milk and biologically relevant functions need the assumption that they are not degraded in the gastrointestinal lumen but absorbed in a functional form. As mentioned above, this may occur and be particularly important in newborn infants. Furthermore, prolonged breastfeeding has been associated with a lower risk of obesity in adulthood in comparison to formula fed infants. The preliminary hypothesis associated this effect to the lower protein content of human milk compared to most infant formula (the early protein hypothesis) [102, 103]. Not only protein content but also the type of proteins present in breast milk are different from those included in formula milks. In this respect, leptin is one of the proteins with a hormone-like function, present in breast-milk and lost during the processing of cow milk, that has been most characterized for its role in prevention of adult obesity. Research in our group during the last few years has been specifically devoted to this field to show the potential role of breast-milk leptin in the prevention of adult obesity [43, 44, 104-107]. In this chapter, a specific section is dedicated to leptin in the context of metabolic plasticity. Below is a description of some other factors that, on the basis of actual knowledge, could also be involved in potential imprinting of newborns towards an adult obese/lean phenotype.

Adiponectin is the most abundant adipose-specific protein present in human milk and serum, circulating at a very high concentration in the latter. Obesity decreases circulating adiponectin and a reduction in adiponectin expression has been associated with insulin resistance. Adiponectin levels in human milk are high in comparison to other hormones, such as leptin and ghrelin. Maternal factors, including duration of lactation, ethnicity and adiposity have been associated with the concentration of adiponectin in breast milk. In contrast with serum levels, a positive association has been found between adiponectin concentration in milk and maternal adiposity. Since prolactin secretion is dampened in obesity and adiponectin is negatively regulated by prolactin, it has been suggested that diminished negative regulation by prolactin in heavier mothers could increase the concentration of adiponectin produced locally in breast tissue and secreted into milk [108]. Reports showing both direct and inverse relationships between milk adiponectin concentration and infant obesity exist [108, 109]. Therefore, more studies are needed to confirm the extent of the association between maternal milk adiponectin and children obesity, as well as the interacting inputs that may affect this relationship. Interestingly, serum adiponectin in breastfed infants is related to the adiponectin concentration in the milk being consumed, suggesting transport across the human intestinal mucosa. In addition, given the biological properties of adiponectin, its presence in breastmilk and the expression of adiponectin receptors in the gastrointestinal tract, it is feasible that milk adiponectin may affect infant growth and development.

Ghrelin has many endocrine and non-endocrine functions: it is involved in energy balance regulation, stimulates food intake and decreases energy expenditure. Ghrelin is an endogenous ligand of the growth-hormone secretagogue receptor (GHSR) and exerts a strong growth-hormone releasing action. Produced primarily in the stomach, it has potent orexigenic and adipogenic activities. Ghrelin is present in significant quantities in human breast milk and there is a direct relationship between milk fat content and ghrelin levels. Although some studies have suggested that milk ghrelin comes from maternal plasma [110], other authors have found evidence that ghrelin in breast milk is most likely synthesized and secreted from

within the breast [111, 112]. Furthermore, there is controversy concerning the relationship between plasma ghrelin and anthropometrical parameters at birth. In the first months of life, ghrelin could exert an important influence on growth, exerting its actions through the receptors found in the gastrointestinal tract [110, 112-116].

Resistin is a cytokine secreted by adipocytes and has been implicated in the development of insulin resistance in animal models, although its role in insulin sensitivity in humans has not been clearly demonstrated. In the perinatal period, resistin does not seem to be directly involved in the regulation of insulin sensitivity or adipogenesis. However, there is a decrease in resistin levels in both milk and serum of breastfeeding mothers, which correlates with hormone status (as prolactin and leptin) and with the concentration of the inflammatory marker C-reactive protein. In addition, resistin concentration has been found to be higher in the serum of breast-fed infants than in either breast milk or their mother's serum. Therefore, resistin, as other breast milk hormones, could be involved in the metabolic development of infants [117, 118].

Insulin is present in human milk in a higher concentration than in commercial fresh cow's milk or infant formula. Insulin content in breast milk is positively related to its concentrations in plasma, and tends to be greater in colostrum and fall gradually in the first weeks post-partum. Milk insulin appears to influence intestinal maturation in the developing gastro-intestinal tract and in animal models and has been associated to activation of mechanisms that suppress the development of autoimmune diabetes [119, 120].

In conclusion, human milk contains a number of peptides relatively resistant to proteolysis and with hormonal/modulatory properties that could contribute positively to the performance of the neonatal gut and, if absorbed, to the newborn. Furthermore, receptors of these peptides are also present throughout the gastrointestinal tract. Therefore, adipokines and hormones involved in the regulation of energy balance and delivered through breast milk to the neonate suggest that maternal inputs are able to transmit timely information regarding food resources and environmental clues, and at the same time predispose the newborn towards a healthier profile.

Oligosaccharides in Human Milk

Human milk is a complex biofluid in which oligosaccharides are the third most abundant solid component (following lactose and lipids). Human milk oligosaccharides (HMO) constitute a heterogeneous group of soluble glycans containing N-acetylglucosamine with a degree of polymerization, and incorporate D-glucose, D-galactose, L-Fucose and N-acetyl neuraminic acid residues. Lactose is found at the reducing end and can be elongated with up to 15 N-acetyl-lactosamine repeating units. Lactose or the polylactosamine backbone can be further sialylated and/or fucosylated. To date, more than 200 HMO have been identified in human milk and their profile indicate that human milk is unique in terms of complexity and content in oligosaccharides. In contrast, infant formula contains only trace amounts of less complex oligosaccharides. Levels of HMO range between 21 to 24 g/L in colostrum and 12 to 14g/L in mature milk. In contrast, the level of milk oligosaccharides in cow's colostrum is 20 to 30 fold lower than in human milk [121-125].

Whether these significant differences cause physiological distinctions between breast-fed and formula-fed infants remain speculative, but may very well be possible considering the putative physiological effects of HMO:

- HMO present in milk are non-digestible by the newborn, withstand the low pH in the gut and resist enzymatic degradation. Therefore, they may undergo bacterial fermentation in the colon and could be considered as prebiotics which modulate microflora ecology [126-129].
- HMO may serve as adhesion soluble ligand analogs, blocking pathogen binding to aerial and intestinal surfaces and protecting breast-fed infants against infections and diarrhoea [130, 131].
- HMO alter expression of glycosyltransferases and, as a consequence, change the glycome of cells [132].
- HMO are partially absorbed intact in the infant's intestine and appear in the urine of breast-fed, but not formula-fed, infants. Therefore, a protective role against urinary pathogens has also been suggested [133-135].

Prebiotic properties of HMO are subject of intensive research and initial characterisation of infant microflora is under development. Bifidobacteria isolated from infants are proficient at capturing and utilizing HMO as a sole carbon source showing metabolic interplay between different species. A model has been proposed in which bacteria such as *Bifidobacterium longum subsp. infantis* captures intact HMO, whereas *B. bifidum* secretes extracellular enzymes prior to translocating lacto-N-biose degradation products and *B. breve* utilizes HMO monosaccharides cleaved by extracellular enzymes secreted by heterologous members of the consortium. Accordingly, a mixed-species transcriptome of the breastfed infant microbiome is enriched for bifidobacterial carbohydrate utilization, suggesting that milk sugars are actively metabolized by phylotypes incapable of utilizing intact HMO under *in vitro* isolation [125, 136].

Therefore, HMO from human origin will contribute to define an infant's microflora different from that originated in formula-fed infants.

Using methods of high-throughput sequencing, it has been described that gut microbiota is able to rapidly shift its membership and representation at the gene content level in response to host adiposity and nutrient environment. Furthermore, specific composition of the gut metagenome has been associated with obesity predisposition in adulthood. Therefore, the potential of breast milk to modulate the composition of gut microflora in infants is highly relevant, taking into account that gut microbiota shapes the host metabolome and this may have an impact in the future phenotype of the infant [137, 138].

Infant Gut Microbiota

The human gut microbiota is dynamic and responsive to dietary changes. Nevertheless, several studies have revealed that microbiota of an individual is more similar over time than to other individual's. However, before the establishment of a stable and diverse microbiota in childhood, this may be different. The fetus is sterile *in utero* and it is rapidly colonized by environmental bacteria at birth and during vaginal delivery, and most of them are derived from the vaginal and fecal microbiota. In fact, the initial bacterial community, even in the

gastrointestinal tract (GI), depends strongly on delivery mode. Lactation is then able to selectively nourish genetically compatible bacteria in infants through its complex array of HMO. The initial microbiota is characterized by low diversity and mainly facultative anaerobic bacteria belonging to Proteobacteria and Actinobacteria (e.g. Bifidobacteria). The gut microbiota then becomes more diverse, and bacteria belonging to Firmicutes and Bacteroidetes are dominant. GI tract microbiota changes throughout life and therefore older adults have substantially different GI tract communities than younger adults. In addition, a number of evidence shows that specific changes in the gut microbiota characterize the obese state and associated metabolic diseases, including diabetes. Knowledge to counteract obesity development by target specific bacterial types with prebiotic compounds though is still insufficient [137, 139-144].

Body weight gain during pregnancy and maternal diet composition (fat and carbohydrate fractions, in particular) are main factors modulating the number of bifidobacteria and lactobacilli in the mother's gut and might be a possible determinant of postnatal obesity development [144, 145].

Diet may favour the development of specific phylum and therefore constitute a major factor in shaping gut microbiota. Gut microbiota may increase energy absorption from the gut by indirect mechanisms (e.g. gut transit time) or direct mechanisms: involving enhanced glucose uptake from the small intestine by a yet unidentified mechanism, fermenting oligo- and polysaccharides to short-chain fatty acids and by modulating lipid absorption. The increased levels of glucose, as well as short-chain fatty acids, can be used for *de novo* lipogenesis. All these metabolic adaptations in lipid metabolism are providing a driving mechanism towards increased adiposity [137, 138].

Thus, programming the gut microbiota in early life by promoting a healthy core microbiome, not necessarily at the level of microbial species, but rather at the level of collective function, may have beneficial effects on host metabolism later in life [121, 125, 137, 144].

Sialic Acid

Newborn infants undergo rapid growth and development, particularly regarding their nervous system. The rate of initial brain growth exceeds that of any other organ or body tissue and by 2 years of age, the brain is about 80% of adult weight. Rapid brain growth places exceptionally high demands on the supply of precursors and nutrients. Sialic acid is a structural and functional component of brain gangliosides and correlates with the amount of DHA and total LC-PUFA in the ceramide tail of brain gangliosides. Sialic acid may be a conditionally essential nutrient in infancy if demand outstrips the rate of endogenous synthesis [146-148].

Several studies show that children who were breast-fed as babies attain higher scores in intelligence tests than those who were bottle-fed [149, 150]. Interestingly, sialic acid present in milk has been shown to enhance brain development, cognition and memory in animals [148, 151].

Sialic acid, an important constituent of human milk, is the generic term for the family of N- and O-substituted derivatives of neuraminic acid. Sialic acid is a key monomeric building block of brain gangliosides and glycoproteins and is part of the diversity of HMO described above. Human milk contains relatively large amounts of sialylated oligosaccharides that are not found in significant quantities in bovine milk or infant formulas. The concentration of

sialic acid in human breast milk varies with genetic, geographic and dietary intake of mothers. The highest concentration of sialic acid is found in colostrum, with a gradual decrease as lactation progresses [152]. In human milk, most sialic acid (approximately 73%) is associated with HMO and this proportion remains fairly constant throughout lactation. In contrast, infant formulas contain most sialic acid bound to glycoproteins (70%). Both glycoprotein (21-28%) and glycolipid (3%) fractions of human milk contain lower levels of sialic acid than that of the free oligosaccharide fraction [146, 148, 152].

Gangliosides are not uniformly distributed within the human body. Ganglioside concentration in brain grey matter is 15 times that of large visceral organs such as liver, lung and spleen and 500 times greater than intestinal mucosa. The mammalian central nervous system has the highest concentration of sialic acid. The majority (65%) is present in gangliosides and glycoproteins (32%) with the remaining 3% as free sialic acid [153]. The finding that babies who had been largely breastfed had significantly higher concentrations of ganglioside-bound and protein-bound sialic acid in the gray matter of their frontal cortex than did the formula-fed infants supports the role of sialic acid and gangliosides in breast milk in human development [154].

Micronutrients

Milk contains micronutrients, compounds which are not synthesized by the human body and are represented by vitamins and minerals, which contribute to vital functions. Traditionally, the mineral fraction has been considered to be composed by macroelements (Ca, Mg, Na, K, P and Cl) and oligo- or microelements (Fe, Cu, Zn and Se). Macroelements are distributed according to their physical-chemical properties, found in a milk aqueous phase or also bound to other milk elements as micelles and carrier proteins (e.g. caseins) [155] or even associated with lipophilic components. Vitamins A, D, E and K are mainly located in the lipid phase and vitamins of group B and C in the aqueous phase.

A large number of micronutrients are either cofactors for enzymes or part of the structure of proteins (metaloenzymes), which are involved in the maintenance of genome stability, including DNA synthesis and repair (Zn, Mg), prevention of oxidative damage to DNA (Zn, vitamin C and E) and methylation status of DNA (folate, vitamins B2, B6 and B12).

In the context of this chapter it is of interest to analyse the potential role of breast milk micronutrients contributing to modify epigenetic marks and how this can have life-long consequences for the newborn, particularly on the potential imprinting of a healthy versus obese phenotype. As mentioned above, one of the most characterised epigenetic mechanisms is DNA methylation on the 5' position of cytosine residues.

Methylation is carried out by specific DNA- and histone methyltransferases, all using S-adenosylmethionine (SAM) as the methyl donor, whose availability is directly influenced by diet. SAM is formed from methyl groups derived from choline, methionine or methyl-tetrahydrofolate. Zn, Se, folic acid and vitamins B6 and B12 are all micronutrients involved in these steps. Furthermore, recovery of the methyl group to form again tetra-hydrofolic acid involves the additional participation of vitamin B2 [156].

Zinc has been identified as the first limiting nutrient in breast milk when anthropometric indicators of growth were correlated with Zn levels in healthy breastfed infants. The high Zn concentrations during the first week postpartum fall consistently as lactation progresses.

MANUSCRITO II

Suckling low-lactose milk is associated with higher fat deposition in rat's adulthood.

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Manuscrito en vías de publicación

Suckling low-lactose milk is associated with higher fat deposition in rat's adulthood

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Abstract

Decreasing the lactose content in dairy products is a preferred strategy to cope with lactose intolerance. This is usually obtained by adding a lactase enzyme to food products intended for human consumption. Production of transgenic mice expressing lactase in the mammary gland to produce low-lactose milk in vivo has been successful and, therefore scaling up to livestock seems feasible. However, more data on the long term effects of the modified milk composition, particularly on progeny are needed. The aim of the study was to perform an in vivo transfection in rat mammary gland of a β-galactosidase gene. Expression of the enzyme would allow pups to suckle breast milk containing a lower lactose concentration and thus to study the metabolic effects of modifying milk composition in both mothers and offspring. Data show the feasibility of transfecting the mammary gland in vivo by the infusion of plasmid DNA encoding a recombinant β-galactosidase gene. Transient activity of the recombinant enzyme was achieved in milk during lactation, allowing 66% of the control levels by day 6 of lactation. However, induction of β-galactosidase in milk was associated with increased maternal body fat content and, more importantly, offspring showed a predisposition to develop obesity in adult life. This data outlines the importance to design long-term studies in order to assess the impact of early nutritional input.

Keywords: lactose; milk; β-galactosidase; obesity

Introduction

Lactose is synthesized in the mammary gland by the lactose synthase complex and constitutes the main sugar present in milk (Jensen 1995). Many adults throughout the world show the symptoms associated with malabsorption of lactose and are lactose-intolerant (NDA 2004; Tunick 2009), because of this, lactose is on the list of food allergens that have to be included in the labelling of food products under European legislation (NDA 2004). Strategies to counteract lactose intolerance without reducing calcium intake include recommendations of dairy products treated to decrease lactose content. However, a recent consensus report recognizes that although lactase-treated products may be tolerated better than non-treated products in intolerant individuals, more research is needed (NIH 2010).

Early attempts to produce lactose-reduced milk have been developed. For example milk containing a lactase enzyme extracted from yeast and fungi was commercialized in 1979 (Tunick 2009) and recently, a food constituent containing a lactase enzyme has been approved by the European Food Safety Authority (NDA 2009). Several experimental methods are able to produce low-lactose milk, including in vitro procedures (Suarez et al. 1995; Tamm 1994; Vilotte 2002), as well as a transgenic mice expressing a lactose-hydrolyzing enzyme in the mammary gland in vivo (Jost et al. 1999). Low-lactose milk can also be produced in vivo by targeting a reduction in the activity of the lactose synthase complex. However, this approach brings about an overall reduction in the sugar content and results in highly viscous milk (L'Huillier et al. 1996; Stinnakre et al. 1994).

The main aim of the present study was to perform an in vivo transfection in rat mammary gland mediated by the infusion of a plasmid encoding a recombinant β-galactosidase gene. Expression of the heterologous protein would allow pups to suckle

60 breast milk containing lower lactose concentration and thus to study the metabolic
61 effects of modifying milk composition in both mothers and offspring.
62

Materials and methods

Preparation of plasmid DNA

The commercially available β-galactosidase expression plasmid pTarget™ (5670 bp) (Promega, Madison, WI) was used. The pTarget™ Vector contains a modified version of the coding sequence of α-peptide of β-galactosidase and carries the human cytomegalovirus immediate-early enhancer/promoter region to promote constitutive expression in mammalian cells. Transformed JM109 (Promega, Madison, WI) bacterial cells were selected and grown. Plasmid was isolated and purified with the Plasmid Maxi Kit (Quiagen, Madrid, Spain). In brief, alkaline bacterial lysate was cleared by centrifugation and the supernatant loaded onto the anion-exchange tip where plasmid DNA selectively binds under appropriate low-salt and pH conditions, allowing removal of impurities by a medium-salt wash, and then ultrapure plasmid DNA was eluted in high-salt buffer. DNA was resuspended in water and concentration determined using a NanoDrop®Spectrophotometer ND-1000 (NadroDrop Technologies, Wilmington, DE, USA).

Experimental animals

Animals were housed under controlled temperature (22ºC) and a 12 h light-dark cycle (light on from 0800 to 2000), and they had unlimited access to water and standard chow diet (Panlab, Barcelona, Spain) containing: 23.5% proteins, 4.3% fat, 3.7% fibre, 5.8% minerals, 51% carbohydrates and 12% water. Female virgin Wistar rats (200-225 g) were mated with male rats (Charles River Laboratories, Barcelona, Spain). All experimental procedures were performed according to both national and institutional guidelines for animal care and use. Day of conception (day 0 of pregnancy) was determined by examination of vaginal smears for the presence of sperm, and from then on female rats were single-caged. Pregnancy evolved without disturbances and four

87 days before the planned parturition, pregnant rats were divided into two groups, control
88 and β-Gal (6 animals/group) according to the subsequent treatment.
89 *Mammary gland in vivo transfection*
90 Plasmid DNA was freshly diluted in PBS to attain a concentration of 1 μg/μl and was
91 then complexed to a DEAE-dextran solution in a ratio 1.39 (Sigma, Saint Louis,
92 Missouri). Transfection mixture was warmed to 37ºC and then injected subcutaneously
93 in the mammary tissue (50 μl in each mammary gland) of the β-Gal group. In vivo
94 transfection was carried out four days before the expected date of parturition under
95 anaesthesia. Control rats followed the same manipulation, but the injection mixture did
96 not contain DNA. Parturition took place between 2-4 days the planned date. The
97 number of pups was adjusted to ten within 24-48 hours after parturition.
98 *Follow up of the animals*
99 After delivery, birth was defined as day 0 of lactation. Food intake, maternal and
100 offspring body weights were periodically recorded. Milk and serum samples of dams
101 were collected on day 6, 12 and 18 of lactation. For milk collection, nursing rats were
102 separated from their pups for 6 h to guarantee that mammary glands were full of milk.
103 Then, dams were anaesthetized and milk was obtained by manual milking of the
104 mammary glands.
105 A sample of blood was also collected from the end of the tail in heparinised tubes and
106 centrifuged at 2500 rpm for 10 min at 4°C to obtain the plasma. All samples were stored
107 at –20 C.
108 Tail blood samples were also collected on day 12 from one randomly selected male and
109 one female from each dam and processed as indicated above.

Body composition of dams (day 10 of lactation) and of a selection of pups was determined (days 12 and 24 of life) by EchoMRI-700™ (Echo Medical Systems, LLC, TX, USA).

At weaning, on day 21 of lactation, the dams plus one male and one female, randomly selected from each dam, were sacrificed and tissue samples collected and weighed. The remaining animals were grouped by sex and maternal treatment and kept in standard housing conditions.

Body weight and food intake of these animals were followed until 5.5 months of age. Body composition was assessed on days 40 and 125 of age by EchoMRI-700™ (Echo Medical Systems, LLC, TX, USA).

Six animals were randomly selected to carry out a test of tolerance to glucose at 125 days of age. A load of 1.5 ml of glucose (1.5 g/kg body weight) was orally given to the animals during the first hour of the beginning of the light cycle after overnight fasting (Lee et al. 2006). Blood glucose concentration was determined from tail blood samples before and at time 0, 30, 60, 120 and 180 minutes postinjection using an Accu Check Sensor (Roche Diagnostics, Barcelona, Spain).

Determinations

Lactose and galactose levels in milk were measured by a colorimetric method (Galactose and Lactose Assay Kit (Biovision, Deltaclon K617-100)). Glucose in milk was determined with a commercial kit (D-glucose UV-method from Roche). Leptin plasma concentrations were assessed by ELISA using the commercial kit Mouse Leptin Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

All data are expressed as the mean ± SEM. Student's test was used to assess statistical differences between control and β-Gal animals. Multiple comparisons were assessed by

repeated measures ANOVA. After 4 months of age, the number of offspring being followed was reduced. Therefore, to consider the maximum number of animals under the statistical analysis of the body weight evolution, ANOVA analysis was performed in two timing sets (from day 0 to 109 and from day 123 to 165) and is indicated in the corresponding legend. The analyses were performed with SPSS for Windows (SPSS, Chicago, IL). Threshold of significance was defined at $P<0.05$ (*).

Results

Dams

Transfection with β-galactosidase gene in mammary gland of pregnant rats caused a transient decrease in the milk concentration of lactose (66% of control values at day 6, $p<0.05$) and galactose (59% of control values at day 6, $p<0.05$). Both sugar concentrations showed control values from day 12 of lactation onwards (Figure 1). In contrast, milk glucose concentration was not altered during the whole period (Figure 1) and the same was seen concerning the levels of total proteins in milk (data not shown). Body weight did not reach statistical significance between control and β-Gal dams during lactation (Figure 2). However, analysis of body composition revealed a higher body fat content in β-Gal dams at day 10 of lactation, in absolute terms (29% higher than controls, $p<0.05$) and also in relationship with the body weight (Figure 3). In accordance with increased body fat content, plasma leptin levels were also higher in β-Gal dams during lactation at day 6 (by 24%) and, specifically at day 12 (by 54%, $p<0.05$), whereas at day 18, recovery of control levels was seen (Figure 4).

At the end of the lactation, blood glucose levels were not affected by the treatment (Table 1). In addition, no significant differences in body weight were observed between groups and this was in correspondence with the weight of the main organs such as liver, mammary gland and stomach. However, a higher retroperitoneal fat mass (1.9 times higher, $p<0.005$) was still found in β-Gal dams with respect to the controls (Table 1) at the end of lactation, in accordance with the higher body fat content previously seen and indicating a specific effect on adipose accumulation (seen also in the sum of the collected fat depots).

Offspring

Transfection with β-Gal gene in mammary gland of the pregnant mothers was not associated with changes in plasma leptin or glucose in offspring at day 12 of lactation (Table 2), plasma glucose was also unaltered at day 20 of age (Table 3). Body and tissues weight at the end of lactation showed no major differences between groups with the exception of heavier BAT in males ($p<0.05$) and slightly heavier liver in females ($p<0.05$) and a more developed eWAT in females from β-Gal dams (1.6 times, $p<0.05$) (Table 3).

After weaning, animals from β-gal treated dams showed higher body weight than the respective controls (Figure 5) a condition that was not accompanied by increased food intake (Figure 6). Furthermore, a clear impact on body fat was observed. Body composition during the perinatal period (measured at days 12 and 24) did not show differences between groups. However, from day 40 and, particularly at adult age (4 months), an increased body fat content was observed in animals from β-Gal dams (25% higher in females ($p<0.05$) and 15% in males) (Figure 7). No disturbances in the homeostasis of glucose were observed after a glucose tolerance test performed in adult life (Figure 8).

Discussion

Some intents to decrease lactose concentration in milk have been proposed as a suitable way to eliminate the problems related to lactose intolerance (Jost et al. 1998; Jost et al. 1999; NDA 2009; Sinha et al. 2007). Lactose may be eliminated in vitro by post-harvest processes, but it has been shown that direct production of milk reduced in lactose is more efficacious (Onwulata et al. 1989). Our interest was to assess the long term nutritional effects in rats of suckling milk synthesised in the mammary gland potentially with lower lactose levels by encoding a recombinant β-galatosidase enzyme.

To develop the low-lactose milk we used a method based on the in vivo transfection of a DNA/DEAE-dextran solution in rat mammary gland, which has been shown to work efficiently in guinea pigs to obtain the expression of the corresponding protein secreted in milk (Hens et al. 2000). The enzyme β-galactosidase was selected because it is used in multiple industrial and biotechnological applications, most of them concerning the removal of lactose from milk products (Husain 2010; NDA 2009).

Our data show the feasibility of transfecting the mammary gland in vivo by the infusion of plasmid DNA encoding a recombinant β-galactosidase gene. Transient activity of the recombinant enzyme was achieved in milk during lactation, allowing lactose levels of 66% the control values, by day 6 of lactation. Total absence of lactose was not desirable because, lactose, together with other sugars and diffusible ions, is responsible for the osmotic pressure of milk (Stinnakre et al. 1994). Therefore, the approach used was enough to produce a significant reduction in lactose, at least during the first third of lactation.

In addition, hydrolysis of lactose by the heterologous enzyme would produce glucose and galactose in proportion to the decrease in lactose content. However, partial reabsorption of those monosaccharides seemed to be produced after lactose hydrolysis, as seen in a mice transgenic model expressing intestinal lactase in milk (Jost et al. 1999). In fact, galactose reabsorption seems to be stronger than glucose and a decrease (59% at day 6) in milk content was also found, whereas concentration of glucose was not altered throughout lactation, which suggests the presence of mechanisms to keep glucose concentration unaltered. No changes in milk protein concentration suggest that the nutritional value of the milk could fulfil the amino acid requirements of growth for offspring.

Unexpectedly, the induction of β-galactosidase in milk was associated with a tendency to increase maternal body fat content (which was also reflected in higher plasma leptin levels). By the end of lactation, a few adipose depots still reflected the excess body fat in the transfected animals. Maybe the availability of more glucose and galactose, because of the hydrolysis of lactose, delayed the mobilization of maternal fat resources for milk synthesis, contributing to the higher fat content in β-galactosidase expressing animals. Interestingly, the growth rate of offspring was not altered during lactation as seen in transgenic mice (Jost et al. 1999). However, longer follow up until adulthood was associated to higher body weight in offspring from transfected animals. Offspring body fat content was not different between groups at mid lactation, but started to diverge after weaning and was completely apparent at 4.5 months of life. Because, no increased food intake was found; other mechanisms affecting the efficiency of nutrient use as metabolic substrates should be involved.

Conclusions

We have shown that in vivo transfection of a reporter gene in rat mammary gland allows a transient expression of a recombinant protein, which may be a very useful approach for potential application in laboratory animals at lower cost and difficulty than the generation of transgenics (Hens et al. 2000; Jost et al. 1999) . The objective to produce a low-lactose milk was nicely achieved and milk containing around lower lactose content than control animals was transiently available to offspring. However, follow up of the animals suggested that this procedure is associated with development of obesity in adult offspring. Therefore, the approach would not be suitable for scaling up in livestock or for human consumption.

Authors' contribution

MS participated in the experimental design of the study, in the animal procedure, carried out molecular and biochemical determinations, participated in the discussion of the results and writing the article. FS and AP conceived the study, participated in its design and coordination and prepared the final version of the manuscript. All the authors read and approved the final version of the manuscript.

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Table legends

Table 1

Body and tissue weight of female rats and blood glucose levels at the end of lactation (day 20)[a,b,c]

[a] Four days before planned parturition, animals were *in vivo* transfected in mammary gland with a gene construct able to express β-galactosidase gene. Then, animals were housed and treated under standard conditions.

[b] Blood and tissue samples were collected at sacrifice on day 20 of lactation.

[c] Data are expressed as the mean ± SEM of 6 animal per group. * = $p<0.05$, from the respective control group (Student's *t* test)

Table 2

Plasma glucose and leptin levels in offspring at day 12 of lactation[a,b]

[a] β-Gal group has been formed with offspring animals from female rats transfected with β-Gal gene in mammary gland at the end of pregnancy. Control group received the same manipulation but the transfection did not contain plasmid.

[b] Data are expressed as the mean ± SEM (n=6)

Table 3

Body and tissue weights of offspring and blood glucose levels at the end of lactation (day 20) [a,b,c]

[a] β-Gal group has been formed with offspring animals from female rats transfected with β-Gal gene in mammary gland at the end of pregnancy. Control group received the same manipulation but the transfection did not contain plasmid.

[b] Blood and tissue samples were collected at sacrifice on day 20 of lactation.

276 [c] Data are expressed as the mean ± SEM of 6 animals per group. * = $p < 0.05$, from the

277 respective control group (Student's *t* test).

278

278 **Figure legends**

279 **Figure 1. Milk sugar composition.** Lactose, galactose and glucose levels were
280 determined in milk at days 6, 12 and 18 of lactation of control and β-Gal transfected
281 dams. Four days before parturition animals were in vivo transfected in mammary gland
282 with a gene construct able to express β-galactosidase gene. Then, animals were housed
283 and treated under standard conditions. Data are expressed as the mean ± SEM of 6
284 animals per group. * = $p<0.05$, from the respective control group (Student´s *t* test).
285

286 **Figure 2. Body weight in lactating dams.** Evolution of body weight of control and β-
287 Gal dams during lactation.
288 Data are expressed as the mean ± SEM of 6 animals per group.
289

290 **Figure 3. Body composition in lactating dams at 10 days of lactation**. A) Body fat
291 content; B) Lean body mass and C) Body fat referred to the body weight of the
292 respective animal. Data are expressed as the mean ± SEM of 6 animals per group. * =
293 $p<0.05$, from the respective control group (Student´s *t* test).
294

295 **Figure 4. Plasma leptin in lactating dams.** Plasma leptin in control and β-Gal dams on
296 days 6, 12 and 18 of lactation. Data are expressed as the mean ± SEM of 6 animals per
297 group.* = $p<0.05$, from the respective control group (Student´s *t* test)
298

299 **Figure 5. Evolution of body weight in offspring.** Data are expressed as the mean ±
300 SEM of 13-20 animals per group from day 3 to day 109 and of 6-10 animals per group
301 from day 123 onwards.
302 ANOVA 109: Repeated measures ANOVA from day 3 to 109 of age.

ANOVA 165: Repeated measures ANOVA from day 123 to 165 of age.

T: Effect of time, S: Effect of sex, G: Effect of group; significance was set at $p<0.05$

Figure 6. Food intake in offspring of β-Gal dams along adulthood. Data are expressed as the mean ± SEM (n= 6).

ANOVA: T: effect of time; S: Effect of sex; significance was set at $p<0.05$

Figure 7. Evolution of body fat content in offspring of β-Gal dams. Data are expressed as the mean ± SEM (n=6 on day 12, n=10 on day 24; n=14-20 on days 40 and 125).

ANOVA 40-125: Repeated measures ANOVA for day 40 and 125 of age.

T: Effect of time, S: Effect of sex, G: Effect of group, significance was set at $p<0.05$

Figure 8. Glucose tolerance test in offspring of β-Gal dams. Data are expressed as the mean ± SEM of 6 animals per group.

ANOVA: T: effect of time; S: Effect of sex; significance was set at $p<0.05$

Table 1

	Control	**β-Gal**
Body weight (g)	315±5	320±8
Mammary gland (g)	15.4±1.6	15.4±1.5
Liver (g)	15.5±0.6	15.4±0.4
Stomach (g)	1.84±0.1	2.03±0.09
eWAT (g)	3.00±0.1	4.40±0.6
mWAT (g)	1.54±0.2	1.64±0.2
rWAT (g)	1.72±0.3	3.26±0.5*
Total white adipose (g)	6.72±0.3	10.1±1.0*
BAT (g)	0.27±0.05	0.26±0.04
Glucose (mM)	6.83±0.61	5.72±0.28

320 **Table 2**

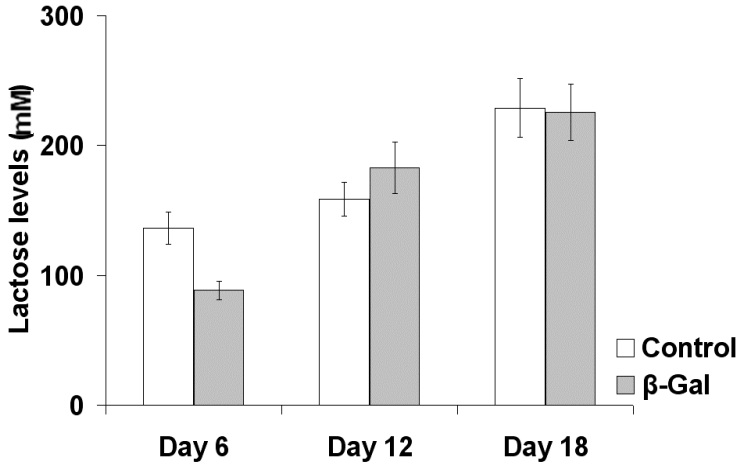
Day 12		Male	Female
Glucose (mM)	Control	5.83 ± 0.39	5.61 ± 0.28
	β-Gal	4.98 ± 0.38	5.00 ± 0.36
Leptin (pg/ml)	Control	969 ± 157	1210 ± 200
	β-Gal	823 ± 135	960 ± 176

321

Table 3

	Male		Female	
	Control	β-Gal	Control	β-Gal
Body weight (g)	49.9 ± 2.1	50.5 ± 2.3	46.8 ± 1.1	50.9 ± 1.8
Liver (g)	2.20 ± 0.20	2.17 ± 0.16	2.01 ± 0.03	2.23 ± 0.09*
Stomach (g)	0.37 ± 0.01	0.35 ± 0.02	0.37 ± 0.02	0.39 ± 0.03
eWAT (mg)	117 ± 9.8	114 ± 18	67.2 ± 7.8	105 ± 12*
mWAT (mg)	262 ± 34	259 ± 21	223 ± 26	297 ± 25
rWAT (mg)	90.7 ± 11.0	84.3 ± 13.8	71.3 ± 5.8	85.0 ± 12.1
iWAT (mg)	378 ± 59.4	315 ± 33.5	305 ± 43	407 ± 95
Total white adipose tissue (mg)	848 ± 94.4	800 ± 61.5	667 ± 43	820 ± 121
BAT (mg)	204 ± 18	264 ± 8*	203 ± 22	249 ± 14
Glucose (mM)	7.60 ± 0.32	7.72 ± 0.31	7.72 ± 0.24	7.55 ± 0.48

322 **Figure 1**



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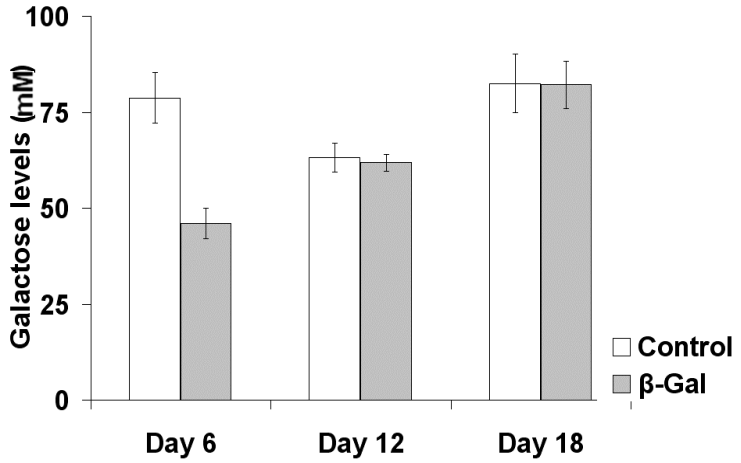

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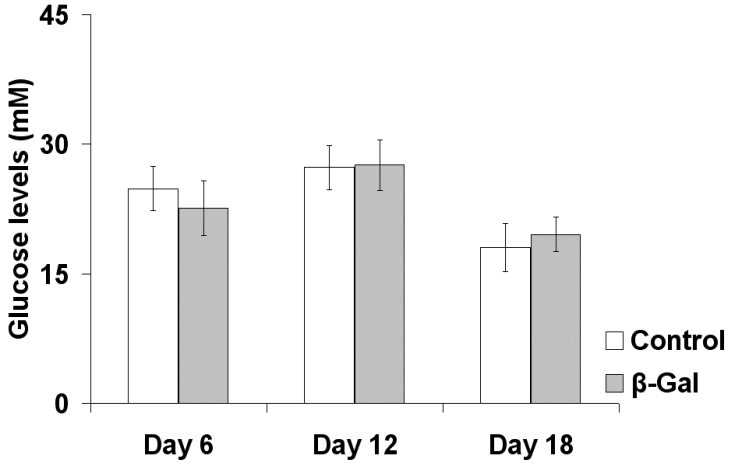

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326 **Figure 2**

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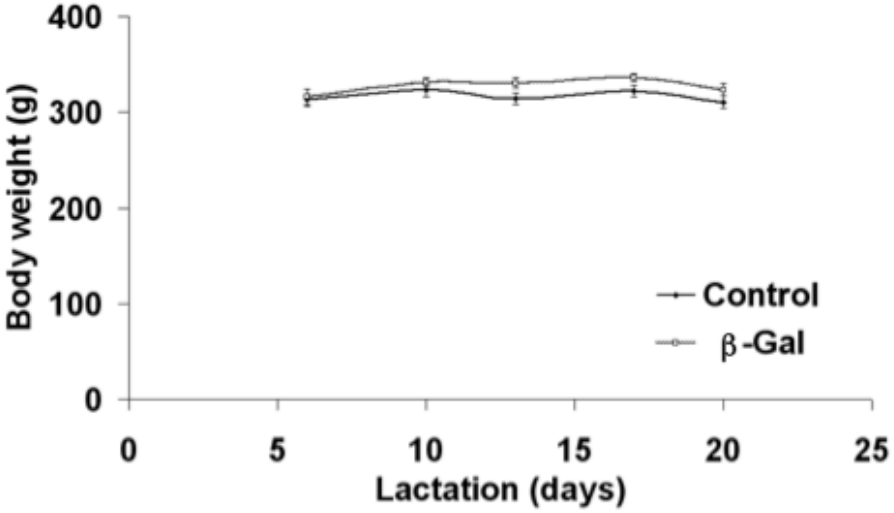
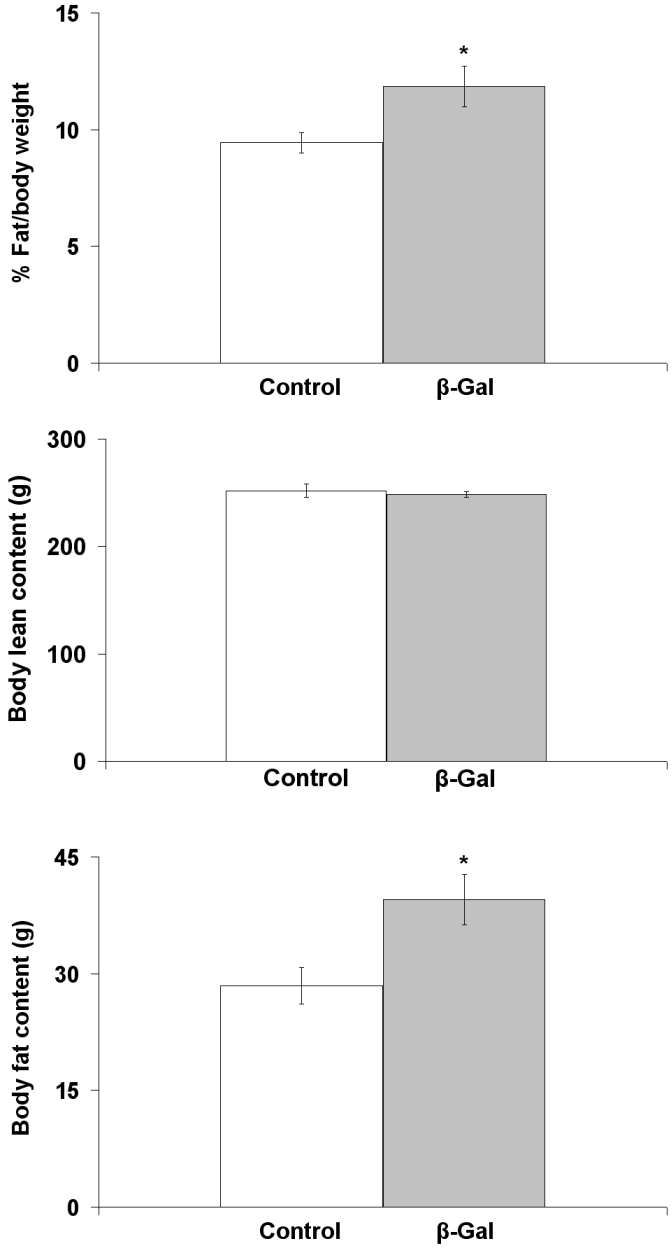
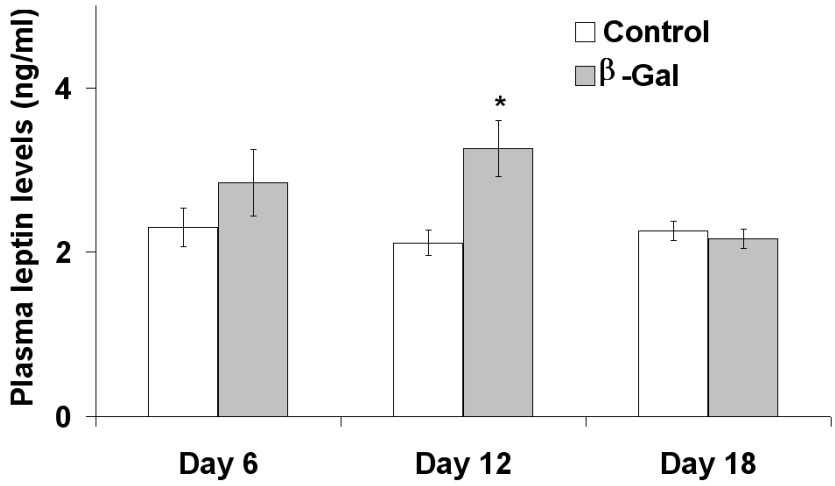


Figure 3


% Fat/body weight
15
10
5
0
*
Control
β-Gal
Body lean content (g)
300
200
100
0
Control
β-Gal
Body fat content (g)
45
30
15
0
*
Control
β-Gal

333 **Figure 4**



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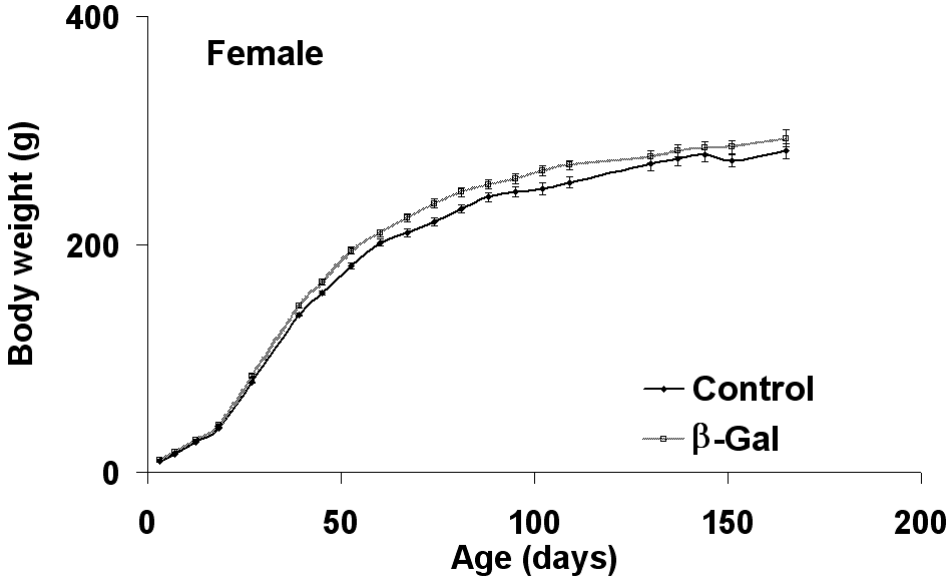
335 **Figure 5**



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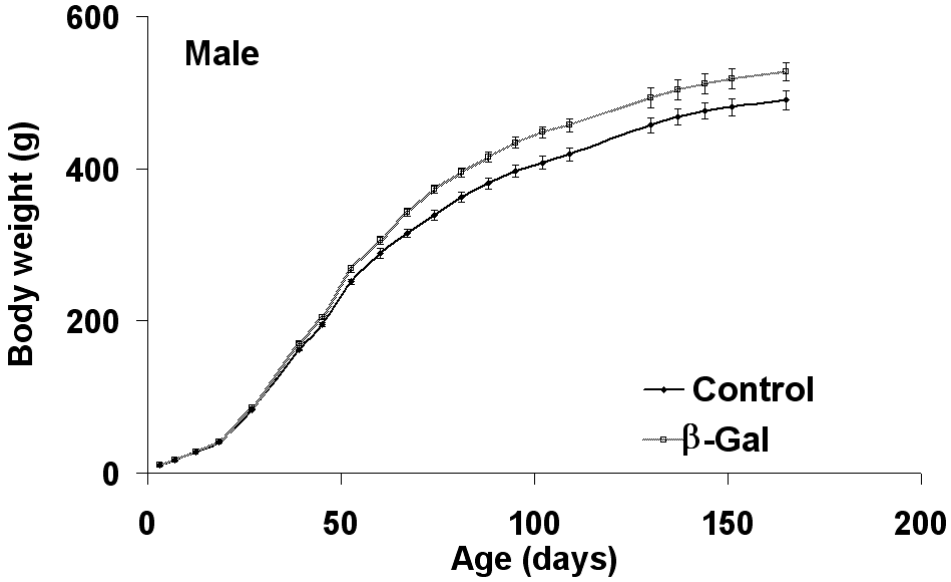

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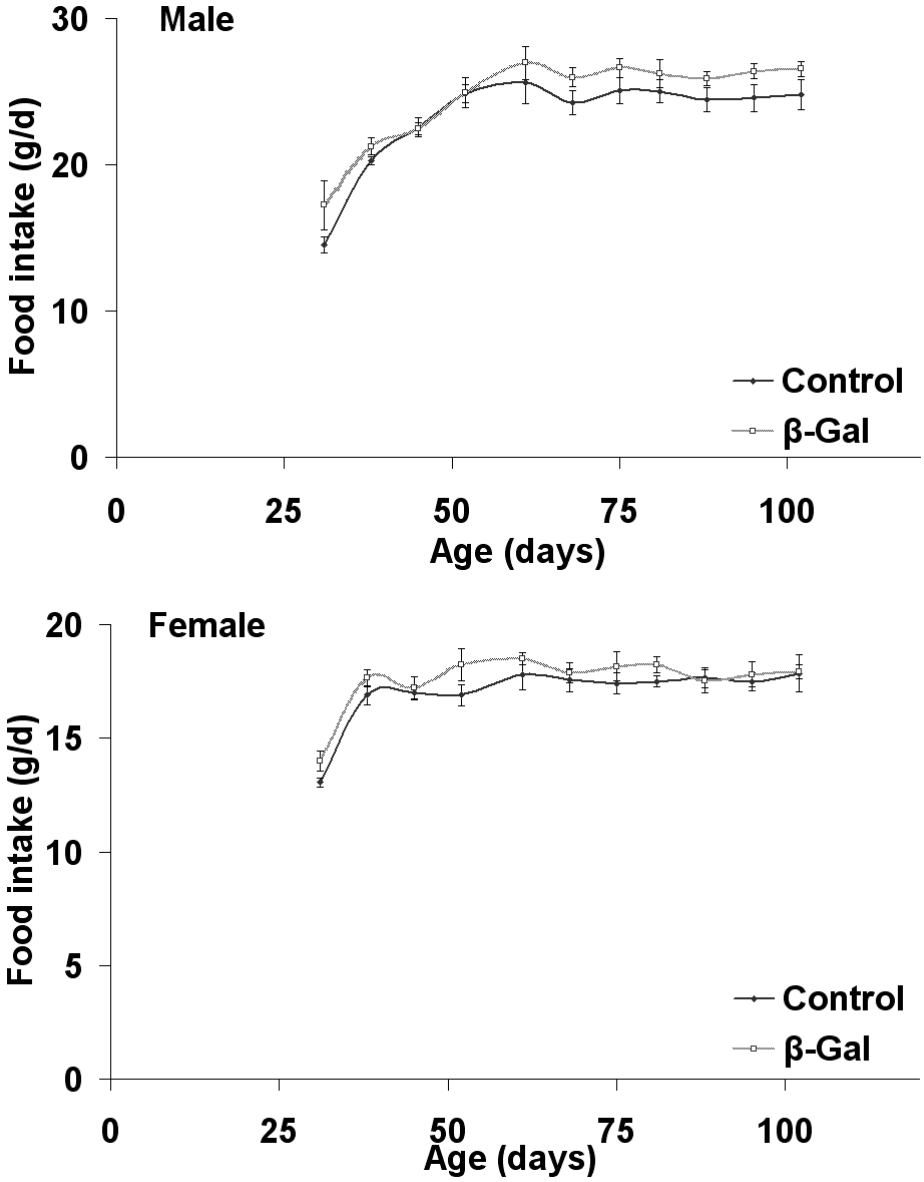
338 **Figure 6**



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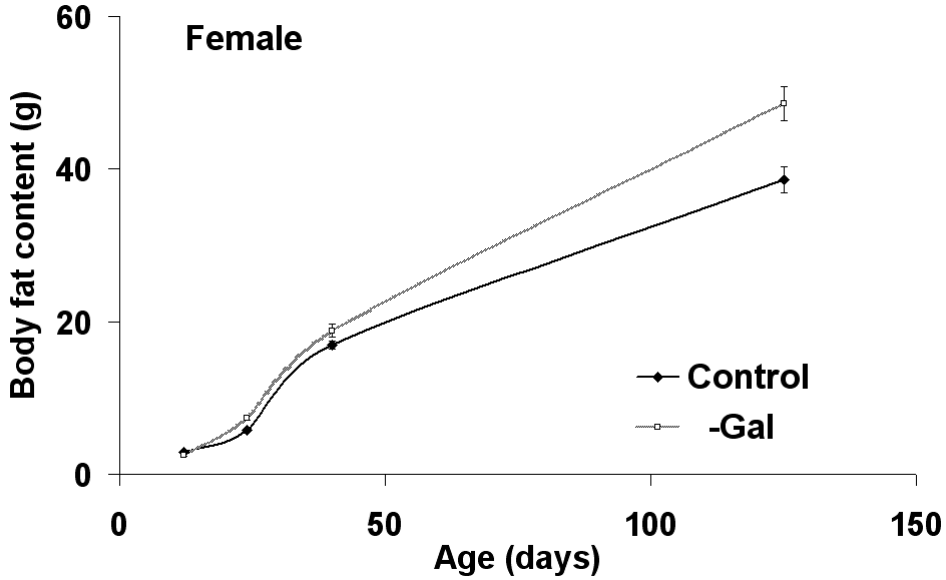
341 ANOVA: T, S

342 **Figure 7**



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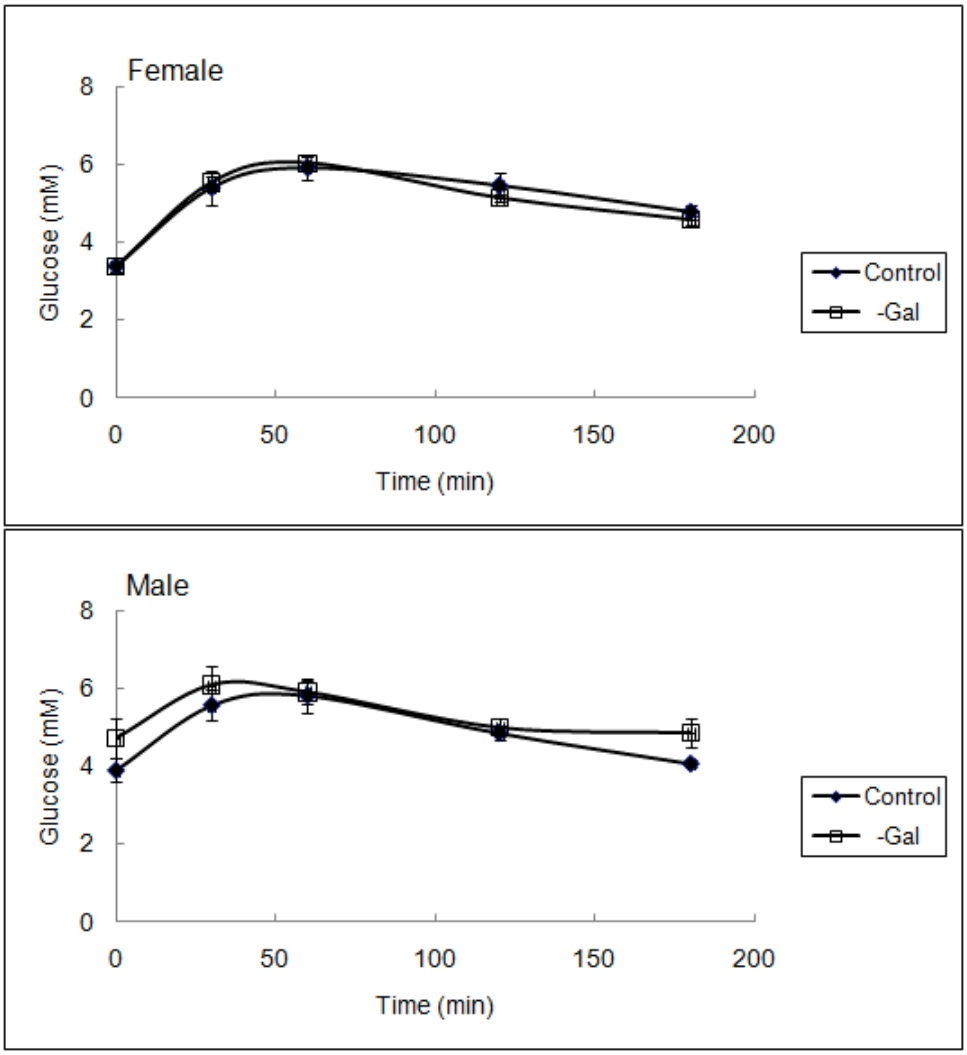
344

345 ANOVA 40-125: TxG, TxS, T, S, G

Abbreviations used

Brown adipose tissue (BAT), white adipose tissue depots: epididymal (eWAT), mesenteric (mWAT), retroperitoneal (rWAT), inguinal (iWAT)

Figure 8



ANOVA: TxS, T

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Reviewers Guide

PART A:

SECTION I

Reviewer's Name:	
E-Mail:	
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Country:	
Manuscript Number:	**AJB-13-110**
Title:	**Suckling low-lactose milk is associated with higher fat deposition in rat's adulthood**
Date Sent to Reviewer:	
Date Expected from Reviewer:	
Area(s) of Specialization (if you wish for your names and affiliation to be uploaded as a reviewer for AJB)	

PART B: ***Reviewer Only***

SECTION II: Comments per Section of Manuscript

General comment:	The main aim of the present study was to perform an in vivo transfection in rat mammary gland mediated by the infusion of a plasmid encoding a recombinant β-galactosidase gene and analyze the metabolic parameters of modifying milk composition in both mothers and offspring. This data outlines the importance of early nutritional input.
Introduction:	
Methodology:	Why did the authors determine the blood glucose concentration before and at time 0 minutes post-injection?
Results:	I think the X axis scale of Figure 8 should be changed to 0,30,60,90,120, 150,180
Discussion:	The novelty of this study was not clearly described. Grammar errors: a low-lactose milk, Some intents to decrease lactose…

SECTION II (Cont.)

Bibliography/References:	
Decision:	Requires Moderate Revision:

SECTION III - Please rate the following: ***(1 = Excellent) (2 = Good) (3 = Fair) (4 = poor)***

Originality:	**3**
Contribution To The Field:	**2**
Technical Quality:	**2**
Clarity Of Presentation :	**2**
Depth Of Research:	3

SECTION IV - Recommendation: (*Kindly Mark With X*)

Accept a its is:	
Requires Minor Corrections:	
Requires Moderate Revision:	***X***
Requires Major Revision:	
Submit to another Publication such as:	
Reject on Grounds of (Please Be Specific):	

SECTION V: Additional Comments

- Please add any additional comments (Including comments/suggestions regarding online supplementary materials, if any):

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PART B: *Reviewer Only*

SECTION II: Comments per Section of Manuscript

General comment:	The manuscript is original. It is the collection of interesting data.
Introduction:	
Methodology:	1. The author did not provide any information regarding permission statement from the concern board using these animals for such experiments. Such statement should be included in the manuscript. 2. This experiment did not provide any confirmatory test showing successful transfection of the desire DNA into mammary gland of the studied animals.
Results:	
Discussion:	1. Some intents to decrease lactose concentration in milk have been proposed as a suitable…..should be written as…… Some intent to decrease lactose concentration in milk has been proposed as a suitable…..

SECTION II (Cont.)

Bibliography/References:	
Decision:	

SECTION III - Please rate the following: ***(1 = Excellent) (2 = Good) (3 = Fair) (4 = poor)***

Originality:	**1**
Contribution To The Field:	**2**
Technical Quality:	**2**
Clarity Of Presentation :	**1**
Depth Of Research:	2

SECTION IV - Recommendation: (*Kindly Mark With X*)

Accept a its is:	
Requires Minor Corrections:	**X**
Requires Moderate Revision:	
Requires Major Revision:	
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Reject on Grounds of (Please Be Specific):	

SECTION V: Additional Comments

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SECTION I

PART B: ***Reviewer Only***

SECTION II: Comments per Section of Manuscript

General comment:	In the current paper transgenic mice expressing lactase in the mammary gland to produce low-lactose milk is investigated in both mothers and offspring. Although paper is good attempt by the author, it needs to be improved before accepting it for publication. The title is not appropriate with the content of the paper. The conclusion does not cover the stated aims. Manuscript contains numbers of figures and tables, which should be reduced to maximum extent.
Introduction:	No comments
Methodology:	The description in "*Follow up of the animals*" section is confusing and unclear to readers. Please rewrite and reorganize it. Page 3, line 5: change "β-galactosidase" to "β-galactosidase (β-Gal)" Page 5, line 3: change "at –20 C" to "at –20°C"
Results:	How to determine the "Fat/body weight", "Body lean content", and "Body fat content" showed in figure 3. Please give references or explanation. What are "eWAT", "mWAT", and "rWAT" in Table 1? Glucose (mM) means (nM/L) or (mM/ml). Please correct display it. Statistical significances are not observed between control group and β-Gal group in Figure 5, 6, 7, and 8, in fact, therefore, they may be deleted from the text. There are no "*" in Figure 1. Figure 2 may be deleted, because there is no difference among groups. Table 2 may be deleted. Table 3: change "(mg)" to "(g)"
Discussion:	Page 7, line 3: change "β-galactosidase" to "β-Gal" Page 8, the last line: change "β-galactosidase" to "β-Gal" Page 9, line 8: change "β-galactosidase" to "β-Gal". The same change is also suitable for other parts in the discussion section.

SECTION II (Cont.)

Bibliography/References:	The literatures cited in this paper are not sufficient, and some of them are too old.
Decision:	I found this manuscript is not recommendable in its current form

SECTION III - Please rate the following: *(1 = Excellent) (2 = Good) (3 = Fair) (4 = poor)*

Originality:	**3**
Contribution To The Field:	**3**
Technical Quality:	**2**
Clarity Of Presentation :	**3**
Depth Of Research:	4

SECTION IV - Recommendation: (*Kindly Mark With X*)

Accept a its is:	
Requires Minor Corrections:	
Requires Moderate Revision:	
Requires Major Revision:	**X**
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SECTION V: Additional Comments

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PART B: ***Reviewer Only***

SECTION II: Comments per Section of Manuscript

General comment:	The idea is interesting. But the discussion of the results needs to be revised.
Introduction:	Good.
Methodology:	Good.
Results:	Minor revision.
Discussion:	Moderate revision.

SECTION II (Cont.)

Bibliography/References:	Good.
Decision:	Minor revision.

SECTION III - Please rate the following: ***(1 = Excellent) (2 = Good) (3 = Fair) (4 = poor)***

Originality:	**2**
Contribution To The Field:	**2**
Technical Quality:	**2**
Clarity Of Presentation :	**3**
Depth Of Research:	3

SECTION IV - Recommendation: (*Kindly Mark With X*)

Accept a its is:	
Requires Minor Corrections:	
Requires Moderate Revision:	
Requires Major Revision:	**x**
Submit to another Publication such as:	
Reject on Grounds of (Please Be Specific):	

SECTION V: Additional Comments

- Please add any additional comments (Including comments/suggestions regarding online supplementary materials, if any):

Questions：

1. I cannot see any obvious difference (p value?) in the body weight and the body fat content between β-Gal group and control group in Figure 5 and Figure 7. So I do not completely agree with the author in that low-lactose milk contributes to developing obesity of the offspring. Is there any similar publication results got by other scientist?

2. According to the author, the possible reason for why the dams in β-Gal group were more fat is because they reabsorbed the glucose and galactose after the hydrolysis of lactose by

β-galactosidase. Then what is the reason for the obesity of the offspring, if they were? I think the offspring has no chance to reabsorb the glucose and galactose.

3. Did the author determine the concentration of plasma galactose of the transgenic mice during lactation? If the transgenic mice will reabsorb more galactose after the hydrolysis of lactose, then the concentration of plasma galactose will be higher than control?

4. Please recheck the legend for Fig. 3.

MANUSCRITO III

Gene expression of mammary gland under L-Leucine treatment during lactation.

Servera M, López N, Serra F, Palou A.

Manuscrito en vías de preparación

IMPACT OF MATERNAL L-LEUCINE SUPPLEMENTATION ON MAMMARY GLAND AND MILK COMPOSITION IN RATS

Authors: Nora López, María Servera, Francisca Serra*, Andreu Palou

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Key words: mammary gland, lactation, milk leptin, leucine

Abstract

Nutritional programming during the perinatal period is a critical step that may affect the propensity to metabolic diseases in offspring. Mother's habits during lactation, as dietary intake and food preferences, may influence milk quality. In addition, milk composition reflects the modulatory processes that take place in mammary gland, the responsible organ of production and secretion of milk. Recently, the essential amino acid leucine has been proposed as a key substrate modulating relevant signalling pathways in energy metabolism, particularly in relation with improvement in energy balance. Therefore, in this study we aimed to characterize the metabolic profile in mammary gland of animals submitted to a diet enriched in L-leucine (2 %) during lactation, and to assess the potential impact on progenie associated to the quality of the milk. Expression of relevant genes in carbohydrate, lipid and protein metabolism has been determined by RT-PCR. The results highlight the relevance of minor nutritional interventions during lactation. Data shown a transient increase in leptin content in milk and relevant impact in genes associated with the synthesis of lactose, casein and lipids. Therefore, the increased leptin content in milk associated with the maternal dietary intervention during lactation could have a beneficial impact on the susceptibility to obesity in adult life of the offspring. However, further research would be necessary to fully characterize the impact observed in significant biosynthetic pathways in mammary gland.

Introduction

Lactation is an important physiological process conditioning offspring growth and development. Metabolic programming effects have been associated with perinatal nutrition and evidences of the impact of lactation on disease susceptibility, particularly on obesity, have been widely demonstrated in experimental animal models (Pico, Jilkova et al. 2011, Picó, Palou et al. 2012). There is no doubt that breastfeeding is the best strategy to nourish infants (Kramer and Kakuma 2004) and among other beneficial effects, lactation in comparison with bottle feeding, has been associated with a reduction in childhood obesity risk (Gale, Logan et al. 2012). At this respect, previous research in our lab has identified the presence of leptin in breast milk, as the compound responsible for programming early prevention to obesity in adult life (Palou and Picó 2009, Palou, Sánchez et al. 2009, Palou, Sánchez et al. 2009).

Lactation is a very high energy demanding process, which requires an increased metabolic rate to accommodate milk production to the newborn demands. Mammary gland needs to deal with the high demand of nutrients for milk production. This is accomplished by shuttling nutrients from other tissues and by upregulation of biosynthetic process in mammary gland, involving lipid synthesis and proteins (Emmett and Rogers 1997, Rudolph, McManaman et al. 2007, Maningat, Sen et al. 2009). At this respect, milk composition reflects the activities of distinct secretion and transport processes of mammary gland and should fit the nutritional requirements of the neonate. Maternal nutrition, including dietary supplements, can influence these processes, may modulate the quality of the milk and therefore growth performance of the breastfed infant (Emmett and Rogers 1997, Chierici, Saccomandi et al. 1999, Muhlhausler, Gibson et al. 2011). Supply of essential amino acids play an important role in the synthesis of milk proteins in mammary gland. Branched-chain amino acids are not only the major components of milk proteins but are also precursors for the synthesis of other amino acids in mammary gland and it has been suggested that dietary supplementation (Lei, Feng et al. 2012). We focussed our interest in the branched-chain amino acid leucine because of its potential role in management of obesity (Layman and Walker 2006) and previous beneficial effects that we have observed in dams supplemented with leucine during lactation (López, Sánchez et al. 2010). In this paper, we have analyzed the influence of dietary leucine supplementation on the expression of genes involved in mammary gland metabolism in order to assess its potential impact on milk composition and therefore in growth performance.

Material and Methods

Animals and experimental design

Twelve virgin female Wistar rats of 189 ± 2g were housed under controlled temperature (22ºC) and a 12 h light – 12h dark cycle (light on from 08:00-20:00). They were adapted for 4 weeks to powder standard chow diet (3000Kcal/kg containing 1.11 g L-leucine/100 g). Then, they were mated and placed in individual cages with free access to water and the powder standard chow diet. At day 1 after delivery, the numbers of pups in each litter was adjusted to 10 per dam (five females and five males when possible). At this time-point, dams were assigned to the control group (C) and continued with the same diet or to the

leucine group (Leu) which consisted with the powder standard diet supplemented with 2% L-leucine (<99% NT, Sigma-Aldrich, Madrid, Spain) (receiving a diet containing 3.11 g L-leucine/100g). The use of animals and the experimental design for this study was approved by the Committee of our University and University guidelines for the use and care of laboratory animals were followed.

Sample collection

For milk collection, nursing rats were separated from their pups for 6h to guarantee that mammary glands were full of milk. Then, dams were exposed to anesthesia and milk was obtained by manual milking of the mammary glands on day 6, 12 and 17 of lactation. Tail blood samples were also collected at the same time.

Leptin concentration in milk and plasma were measured with a Mouse Leptin Enzyme-linked Immunosorbent Assay (ELISA) Kit (R&D Systems, Minneapolis, Min, USA). Adiponectin in milk samples was determined a Adiponectin (Rat, mouse) ELISA Kit (Phoenix Pharmaceuticals, Inc, Burlingame, USA) and in plasma with a Rat total Adiponectin/Acrp 30 (quantikine ELISA) kit (R&D Systems, Minneapolis, Min, USA). Milk lactose content was measured by a colorimetric method (Lactose Assay Kit (Biovision, Deltaclon K624-100)) and total protein concentration by the method of Bradford (Bradford 1976). T3 concentration in plasma samples was measured with an enzyme-linked immunosorbent Assay Kit for Rat Triiodothyronine (T3) (Uscn Life Science Inc, Houston, USA). Commercial enzymatic colorimetric kits were used for the determination of plasma and mammary gland triglyceride and glycerol levels (Triglyceride (INT) 20, Sigma Diagnostics, St Louis, MO, USA) and non-esterified fatty acid (NEFA) (Wako Chemicals GmbH, Neuss, Germany). Previous to mammary gland determinations, an extraction procedure was followed (Rodríguez-Sureda and Peinado-Onsurbe 2005) .

At weaning (day 21) C y Leu animals were sacrificed and samples of mammary gland were collected, snap frozen in liquid nitrogen and stored at -80°C for analysis.

RNA extraction and RT-qPCR analysis

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

Real-time PCR was used to measure mRNA expression levels of FABP3, FABP4, CAV1, Slc27a1, Slc27a3, SCD1, SCD2, Acly, ATGL, HSL, GLUT1, CS, Slc25a1, PC, InsR, IRS1, IRS2, G6PDH, LAT1, CAT1, Lalba, Csn1s1, Csn1s2, Csn2, Csn3, CLOCK, ARNTL, leptin, adiponectin, UCP2, Xbp, Xbp spliced, SERBP1c and CEBPα in mammary gland; 0,25 µg of total RNA (in a final volume of 12.5 µl) was denatured at 90°C for 1 min and then reverse transcriptase to cDNA using murine leukemia virus reverse transcriptase (Applied Biosystem, Madrid, Spain) at 42°C for 60 min and 99°C for 5 min in a Applied Biosystems 2720 Thermal Cycler (Applied Biosystem). Each PCR was performed from diluted (1/5) cDNA template, forward and reverse primers (10 µM each) and Power SYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA). All primers were

obtained from Sigma Genosys (Sigma Aldrich Química SA, Madrid, Spain) and their sequence is listed in Table 1. Real-time PCR was performed using the Applied Biosystems StepOnePlus TM Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 min at 95°C, followed by a total of 40 two-temperature cycles (15s 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 2.0). Relative gene expression numbers were calculated as a percentage of control animals, using the $2^{-\Delta\Delta Ct}$ method with the β-actin gene as a reference gene (Pfaffl 2001).

Determination of miRNA

For miRNA measurements, RNA was first reverse-transcribed and then amplified using the specific primers and probes provided with the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems). The following conditions were used: 16° for 30 min, 42º for 30 min and 85º for 5 min. 2μl of miRNA-specific cDNA from this reaction was amplified with the TAqMan Universal PCR master mix and the respective specific probe provided in the TaqMan MicroRNA Assay (Applied Biosystems). The targeted miRNA assay sequences are shown in Table 2. PCR was performed in the same Applied Biosystems StepOnePlus ™ Real-Time PCR System (Applied Biosystems) as previously described. Relative quantification of a target gene was calculated based on efficiency and the crossing point deviation of an unknown sample versus a control, and expressed in comparison to a reference gene used to normalize cDNA (U6 small nuclear RNA). miRNA expression levels were expressed relative to the values found in the control group.

Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis was performed with SPSS for Windows (SPSS, Chicago, IL). Threshold of significance was defined at $^{*}P<0.05$. Repeated-measures analysis of variance (ANOVA) was used to determine differences along lactation associated with the treatment. For those single time-point variables, mainly gene expression, Student's *t*-test was used to compare the mean differences between groups.

Results

Leucine supplementation increases leptin levels in milk

A transient increase in milk leptin levels was found in Leu dams (at day 12 of lactation) (Figure 1). This was associated with a tendency to increase plasma leptin levels along lactation although it did not attain statistical significance. This pattern could not be associated with an induction of leptin expression in mammary gland, although this was determined at day 21, when leptin levels in Leu supplemented animals have already recovered control values. Concerning adiponectin, although changes were not statistical significant, milk levels of adiponectin seemed to increase at the beginning of lactation (Figure 1) and since then, tended to be lower than in controls. In contrast, plasma levels showed a decrease along the lactation, which was statistical significant the last two days studied (12 and 17). Accordingly, expression

of adiponectin in mammary gland was also diminished although it did not attain statistical significance. The related UCP2 gene also was not differently expressed between C and Leu animals. T3 levels were also determined in plasma and no differences were found between groups (data not shown).

Expression of casein 1α is reduced in Leu animals

We determined the expression of genes related to the transport of amino acids in mammary gland to assess the influence of Leu supplementation on shuttling machinery. Despite of promoting greater availability of dietary Leu, expression of the transporter LAT1, responsible for both aromatic and branched chain amino acids, including leucine, was not altered (Figure 2). The same was observed for the expression of the large neutral amino acid transporter (ASC) and the cationic amino acid transporter (CAT1).

Although total protein levels in milk were unaltered by the supplementation (data not shown) analysis of major protein synthesis genes indicated that CSN1S1, responsible for the αS1-casein synthesis, was reduced in Leu animals (48% of the control value) (Figure 2). This was a further specific effect because expression of other milk protein genes as casein αs2-like A (CSN1S2A), β-casein (CSN2) and κ-casein (CSN3) were not different between groups (Figure 2).

Supplementation of Leu decreased GLUT1 and L-alba expression, as well as milk lactose content

We next checked the influence of Leu supplementation on mammary gland carbohydrate handling. A significant decrease (29% of the control value) in mRNA levels of glucose transporter 1 (GLUT1) was found in mammary gland of Leu group; whereas a high variability and no significant differences were found in the expression of the insulin-dependent GLUT4 receptor (Figure 3). Concerning lactose, a significant decrease in mRNA expression of α-lactalbumin (Lalba) (65% of the control value) was found in mammary gland of Leu group (Figure 3) which was accompanied with lower levels of lactose in milk (29% of the control values, p=0.056).

Expression of lipid metabolism genes showed lower levels by Leu supplementation

Gene expression associated with lipid transport was unchanged in Leu-fed animals, this was the case for the fatty acid binding proteins 3 (FABP3) and 4 (FABP4), the long-chain fatty acid transporter protein 1 (Slc27a1) and 3 (Slc27a3) (Figure 3). No differences were observed between groups in caveolin 1 expression (CAV1) or with triglyceride handling associated with the expression of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). However, a decrease in desaturase expression SCD1 (not statistically significant) and SCD2 was observed (21% and 61% respectively of the control values). ATP-citrate synthase (Acly) mRNA levels were also decreased (51% of the control values) (Figure 3) in mammary gland of Leu group.

Minor impact of leucine supplementation is found in other genes of metabolism

Regarding genes related to pentose phosphate pathway, no significant changes were found in mRNA levels of G6PDH between mammary gland depot of control and treated group (Figure 4). A significant decrease in pyruvate carboxilase (PC) mRNA levels was found in Leu group (62% of the control group) (Figure 4), whereas the expression levels of citrate synthase (CS) and the citrate transporter (Slc25a1) were unchanged in the Leu group. Respect to genes associated with insulin response and signalling, no significant differences were found between groups in all of the genes studied: insulin receptor (Insr), substrate 1 and 2 (IRS1 and IRS2) (Figure 4). We checked one gene related to circadian rhythms (CLOCK), which was not altered by treatment (Figure 5), no involvement of endoplasmic reticulum stress was detected at the level of expression of X-box binding protein 1 (Xbp1) and the spliced form (Xbp1 spliced). No changes were found in the transcription factors SERBP1c and CEBPa (Figure 5) and other genes as CIDEA (cell death inducing-DFFA-like effector a), hexokinase, STAT3, STAT5 (signal transducer and activator of transcription 3 and 5) or TGFb (transforming growth factor beta) (data not shown). Finally, there was not any significant difference in miRNA levels analyzed in mammary gland (Figure 5).

Discussion

The main function of mammary gland is milk production to allow the breeding and to ensure success in growth and development of newborn, which depends largely on the quality of breast milk (Prentice 2011, Rodriguez-Cruz, Sánchez et al. 2011). Several adaptations occur during gestation in mammary gland, in order to set up lactation (Peaker 1976, Peaker 2002, Tygesen, Nielsen et al. 2008). Breast milk contains all nutrients and substances needed for successful developmental progression of the newborn. Although maternal nutritional status as well as maternal diet during lactation both can influence the composition of breast milk (Rodríguez-Cruz, Sánchez et al. 2009). However, mammary gland itself has some potential to minimize deleterious consequences for offspring on milk composition (Coward, Paul et al. 1984, Quinn, Largado et al. 2012).

During lactation, mammary gland increases nutrients demands in order to fulfil the milk production requirements. Among them, amino acids have a great importance, because of their fundamental role in the synthesis of milk proteins (Alemán, López et al. 2009). Particularly, leucine is the most abundant amino acid in tissues and food proteins and, as an essential amino acid, can only be obtained from diet (Wu 2009). A large number of reports describe the positive effects of leucine, particularly in promoting protein synthesis (Bolster, Vary et al. 2004, Matsuzaki, Kato et al. 2005, Layman and Walker 2006, Appuhamy, Knoebel et al. 2012), or contributing loss of body fat and to reduce the loss of lean tissue (Zhang, Guo et al. 2007, Chen, Simar et al. 2012, McAllan, Cotter et al. 2012). Furthermore, leucine intake in lactating rats is able to modulate their body composition through a leaner phenotype by a mechanism involving modulation of orexigenic neuropeptides (López, Sánchez et al. 2010).

In this stuy we have found that maternal leucine supplementation induced higher leptin levels in milk, which could have a potential role in the prevention of adult obesity, since leptin has a relevant role in regulation of energy balance. This hormone, naturally present in milk, has been described as the element responsible for some of the beneficial effects of breast milk (Palou and Picó 2009) and specially it is involved in prevention of adult obesity

(Palou, Picó et al. 2011). Maternal leucine supplementation has also an impact on adiponectin, another modulatory protein of adipocyte-origen. Plasma adiponectin were decreased and these results contrast with previous studies in obesity-induced in adult rats where leucine supplementation increases adiponectin plasma levels (Torres-Leal, Fonseca-Alaniz et al. 2011). This may indicate specific adaptation in lactation and although maternal plasma levels decreased, the adiponectin milk content was unaltered, indicating that mammary gland was able to cope with potential changes of this endocrine function protein.

An unexpected finding was the maintenance of the level of expression of amino acids transporter genes, including the responsible for leucine, which did not show significant differences in the mammary gland of treated animals. It is known that these genes are not expressed similarly during pregnancy and lactation (Alemán, López et al. 2009). Furthermore, amino acid uptake depends on the requirements of the mammary gland, which can modulate expression of these transporters. Our data suggest that leucine supplementation did not have a great influence in the mRNA expression of the amino acid transporters. Therefore, associated with a higher leucine intake, milk leucine content was also higher in the supplemented dams (between 1,7 and 2-fold in comparison with controls). In addition, total protein levels in milk were not altered, but synthesis of specific proteins was reduced. Data suggest that even though mammary gland was not adjusting amino acid transporters with treatment, and consequently milk seems to be richer in amino acids; levels of caseins, the main milk protein (Recio, Pérez-Rodríguez et al. 1997), do are modulated by mammary gland gene expression reflecting the effects of treatment. Furthermore, this could determinate de milk quality, since this family of acidic phosphoproteins are stored and secreted as stable calcium phosphate complexes (micelles) (Dalgleish and Corredig 2012) and they contribute to the stability of protein micelles in milk; in addition, Csn1s1 has a role in formation and transport of caseins in the secretory pathway (Le Parc, Leonil et al. 2010).

Regarding carbohydrates, lactose is synthesized in mammary gland by the lactose synthase complex and constitutes the main sugar present in milk (Jensen 1995). Synthesis of this lactose from glucose and UDP-galactose takes place in the trans-Golgi, catalyzed by lactose synthase, and the mammary specific protein α-lactalbumin. The presence of the cofactor, α-lactalbumin, is rate limiting for lactose synthesis (L'Huillier, Soulier et al. 1996, Soulier, Lepourry et al. 1997). Our data shown that gene expression of Lalba (α-lactalbumin) in mammary gland was lower in Leu rats than in controls; this was reflected with a reduction in the concentration of lactose in milk of the Leu rats. Amount of lactose in milk acquires great importance, due to some individuals show symptoms associated with malabsorption of lactose (called lactose-intolerant) (NDA 2004, Tunick 2009). Therefore, this would need further research, particularly looking for a potential application in the definition of a novel strategy to decrease lactose content in milk.

Furthermore, mRNA levels of GLUT 1, the low-level glucose transporter , were also lower in treated animals. Glucose requirements increase during late pregnancy and even more in lactating mammary glands. Major adjustments in glucose production and utilization are undertaken to canalize glucose preferentially to mammary gland respect to other tissues as maternal liver, adipose tissue or skeletal muscle. In pregnant animals, these responses are exaggerated by moderate undernutrition and are mediated by reduced tissue sensitivity and

responsiveness to insulin, associated with decreased tissue expression of the insulin-responsive facilitative glucose transporter Glut4 (Bell and Bauman 1997). However treated animals shown unaltered levels of Glut4 and of other genes related to insulin regulation like insulin receptor (InsR) and their substrates (IRS1 and IRS2), this is accompanied by a decrease in GLUT 1, which is not sensitive to insulin. These data would suggest that the reduction in glucose uptake would contribute to the reduced lactose synthesis in mammary gland.

Finally, we found important changes in gene expression of lipid metabolism. Mammary gland is one of the three major lipid-synthesizing organs in the body, together with liver and adipose tissue (Wakil and Abu-Elheiga 2009). The regulation of lipid synthesis as well as that of lactose in the mammary gland, occur at the level of mRNA expression (Rudolph, McManaman et al. 2007). Concerning genes involved in lipid metabolism, Leu supplementation decreased mRNA levels of SCD2 and ACLY, key genes in fatty acid synthesis in mammary gland and this was accompanied by lower expression of pyruvate carboxilase (PC). Slc25a1 is required, together with ACLY for the conversion of glucose in fatty acids; and SCD2 inserts a single double bond into synthesized or preformed fatty acids (Ntambi, Miyazaki et al. 2004, Rudolph, Monks et al. 2010). Although there was no significant differences in others genes related to fatty acid metabolism and transport, like FABP3 (fatty acid binding protein 3), CAV1, or ATGL, a tendency of decrease was observed, supporting the fact that mammary gland may be decreasing lipid biosynthesis pathways.

In conclusion, dietary maternal supplementation with leucine during lactation caused a transient increase in leptin levels in milk; which would be associated with a better nutritional programming in the context to avoid adult obesity. However, the impact of leucine supplementation is also affecting gene expression in mammary gland and involved a decrease in lactose synthesis and milk lactose content as well as a decreased casein synthesis and in lipid metabolism genes. Altogether indicates the relevance to conduct further studies to assess the impact in adult life of maternal Leu supplementation and to contribute to clarify the underlying mechanisms.

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Table 1. Nucleotide sequences of the primers used to analyze gene expression in mammary gland

GENE	FORWARD PRIMER (5' TO 3')	REVERSE PRIMER (5' TO 3')
ACLY	GGGTTACTCCCGACACAGAC	CGAAGGGCTCAATCAGAAAG
ADIPONECTIN	GCTCAGGATGCTACTGTTG	TCTCACCCTTAGGACCAAG
ARNTL	ACTTCGCCTCCACCTGTTC	CCGTATTTCCCCGTTCACT
ASC	TCTGCCTCTTGTCTATTTTGCTT	ATCCTCTTGTCCACGCCATT
ATGL	TCGTATCACCAACACCAGCA	CCCTCCAGTCCTCTCCTCA
CAT1	CACAGGGGAAGAAGTCAAGAA	CAGGCAGAAGTAGGGCATCA
CAV1	AGACGAGGTGAATGAGAAGCA	CCGAAGATGGTAGACAGCAAG
CEBPA	AGCCGAGATAAAGCCAAACA	CCTTGACCAAGGAGCTCTCA
CLOCK	CGCAGAATAGCACCCAGAG	GGCAAAAGTAGGATAGGCAGTC
CS	ACTCATCCTGCCTCGTCCTT	CTCTTCCCCACCTTTAGCC
CSN1S1	CTTATCCTCACCTGCCTCGT	CACTTGCCGTTGCTTGTTC
CSN1S2	AGGGCAATGGTGTGTTCTTC	CACTGAGGTGCTGGTGGACT
CSN2	ATCCTTGCCTGCCTTGTG	TGTTTTGTGGAATGGGACTG
CSN3	CAAAACCCAGACTCAAACTGC	GGCTAACAGGCACAGAGGTC
FABP3	AGGAAGGTCAAGTCGGTCGT	TGGGTGAGAGTCAGGATGAG
FABP4	TTGTGGGGACCTGGAAACT	TCCTGTCATCTGGGGTGATT
G6PDH	CGAACCACATCTCCTCTCTG	ATCAAAATAGCCCCCACGAC
GLUT1	GCCCCCGCTTCCTGCTCATC	CCCGCATCATCTGCCGACCC

Table 1. (continuation)

GENE	FORWARD PRIMER (5' TO 3')	REVERSE PRIMER (5' TO 3')
HSL	TCACGCTACATAAAGGCTGCT	CCACCCGTAAAGAGGGAACT
INSR	CTCCTGGGATTCATGCTGTT	GTCCGGCGTTCATCAGAG
IRS1	GCAACCGCAAAGGAAATG	ACCACCGCTCTCAACAGG
IRS2	TAGCCACAGGAGCAACACAC	CCATCCCCAGAGACCCTAC
L-ALBA	TGATGCGTTTTGTTCCTCTG	CTGTGCTGCCATTGTTCTTG
LAT1	CCTGCCTCTGCGTGCTACT	CCCTTGTCCTATGTCCTTTCC
LEPTIN	TTCACACACGCAGTCGGTAT	AGGTCTCGCAGGTTCTCCAG
PC	GTTCCCCCGACCTCCCACT	AGCCCCTTCCCAATACTCAC
SCD1	ATCCCCTCCTCCAAGGTCTA	CGGGCCCATTCATATACATC
SCD2	TTTCTCATCATCGCCAACAC	CTTTCCGCCCTTCTCTTTG
SERBP	CCCACCCCCTTACACACC	GCCTGCGGTCTTCATTGT
SLC25A1	GTGGCAGAGGCAGTGGTAGT	ACATAGGCTTGTTGGGGTTGT
SLC27A1	CAGGAGTGGAGGGGAAAG	CAGAAGACGCAGGAAGATGG
SLC27A3	CCGAGCAAGGCAGAATGT	TCAAGGAGAAGGGGAAGATG
UCP2	GGTCGGAGATACCAGAGCAC	ATGAGGTTGGCTTTCAGGAG
XBP	TCCGCAGCACTCAGACTATGT	ATGCCCAAAAGGATATCAGACTC
XBP SPLICED	GAGTCCGCAGCAGGTG	GTGTCAGAGTCCATGGGA

Table 2. Nucleotide sequences of the miRNA analyzed in mammary gland

miRNA	SEQUENCES (5' TO 3')
103	AGCAGCAUUGUACAGGGCUAUGA
107	AGCAGCAUUGUACAGGGCUAUCA
143	UGAGAUGAAGCACUGUAGCUC
221	AGCUACAUUGUCUGCUGGGUUUC
222	AGCUACAUCUGGCUACUGGGU

Table 3. Lipid metabolites in Leu-supplemented dams

	Control	Leucine
Mammary gland		
Glycerol (mg/g)	3.47 ± 1.31	3.20 ± 0.97
Triglycerides (mg/g)	13.98 ± 2.03	15.25 ± 4.70
NEFAs (µmol/g)	14.58 ± 1.6	42.6 ± 2.6**
Maternal plasma		
Glycerol (mg/ml)	0.287 ± 0.023	0.227 ± 0.027
Triglycerides (mg/ml)	0.425 ± 0.073	0.417 ± 0.054
NEFAs (mM)	0.685 ± 0.062	0.659 ± 0.077

Levels of lipid metabolites in mammary gland and plasma were determined at weaning in dams fed with control or leucine-supplemented diet.

Data are mean ± SEM of four-six animals per group. Statistical significant differences were assessed by Student's *t* test, * = $p<0.05$; ** = $p<0.01$.

LEGENDS TO FIGURES

Figure 1. Milk and plasma leptin and adiponectin content and mRNA levels of genes involved in energy balance in mammary gland of lactating rats receiving a dietary supplementation of L-leucine (2%) during lactation. Leptin and adiponectin levels were measured by ELISA and mRNA levels by real-time PCR and expressed as a percentage of the value of control rats.

Results are mean ± S.E.M. of 6–8 animals per group.

Repeated-measures analysis of variance (ANOVA) was used to determine differences along lactation associated with the treatment. For gene expression, Student's *t*-test was used to compare the mean differences between groups.

* = *P<0.05* by Student's *t*-test, comparing Leu animals with controls

Figure 2. mRNA expression of genes associated with casein synthesis and amino acid transport in mammary gland of lactating rats receiving a dietary supplementation of L-leucine (2%) during lactation. mRNA levels were measured by real-time PCR and expressed as a percentage of the value of control rats.

Results are mean ± S.E.M. of 6–8 animals per group.

* = *P<0.05* by Student's *t*-test, comparing Leu animals with controls

Figure 3. mRNa expression of glucose transporter genes and α-lactalbumin in mammary gland and lactose content in milk in lactating rats receiving a dietary supplementation of L-leucine (2%) during lactation. mRNA levels were measured by real-time PCR and expressed as a percentage of the value of control rats. Lactose content was measured by a commercial colorimetric assay.

Results are mean ± S.E.M. of 6–8 animals per group.

* = *P<0.05* by Student's *t*-test, comparing Leu animals with controls

Figure 1

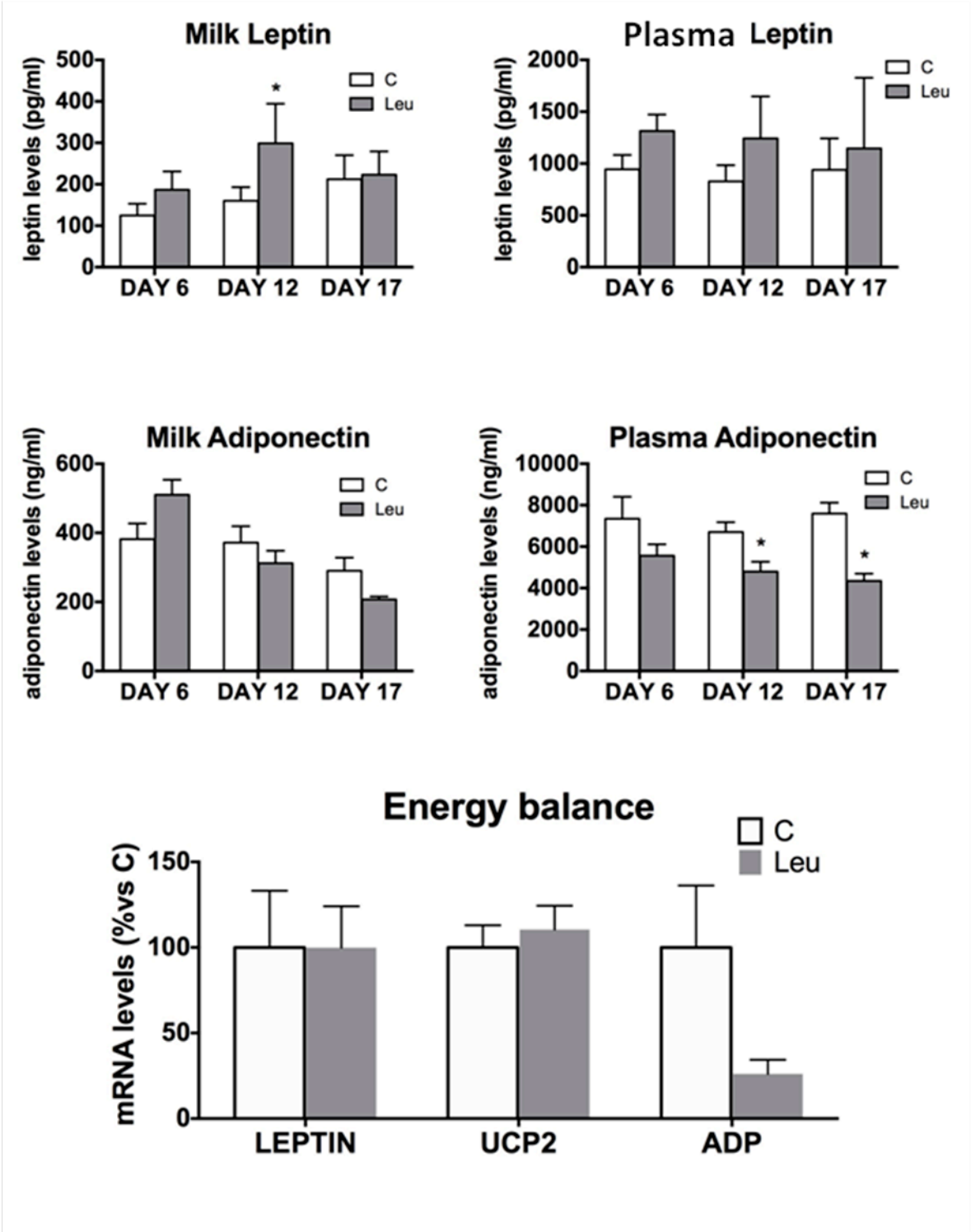

Milk Leptin
leptin levels (pg/ml)
500
400
300
200
100
0
C
Leu
*
DAY 6
DAY 12
DAY 17
Plasma Leptin
leptin levels (pg/ml)
2000
1500
1000
500
0
C
Leu
DAY 6
DAY 12
DAY 17
Milk Adiponectin
adiponectin levels (ng/ml)
600
400
200
0
C
Leu
DAY 6
DAY 12
DAY 17
Plasma Adiponectin
adiponectin levels (ng/ml)
10000
8000
6000
4000
2000
0
C
Leu
*
*
DAY 6
DAY 12
DAY 17
Energy balance
mRNA levels (%vs C)
150
100
50
0
C
Leu
LEPTIN
UCP2
ADP

Figure 2

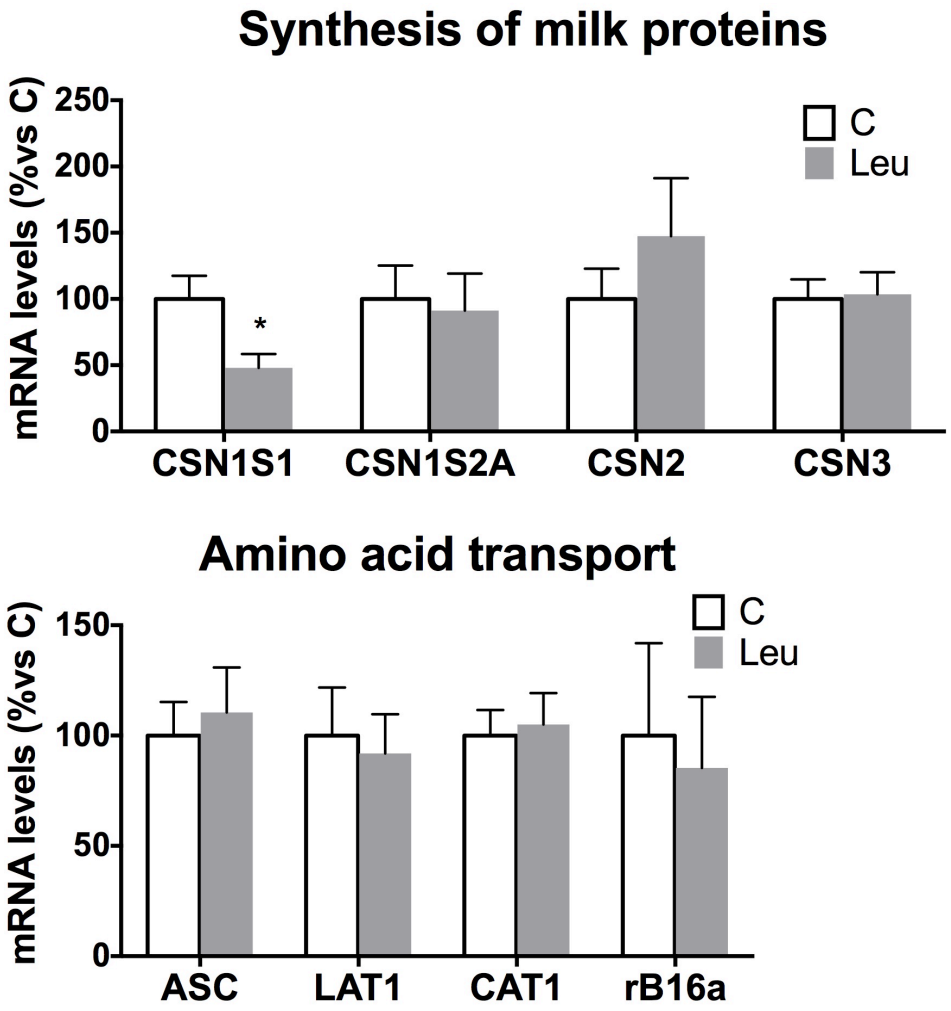

Synthesis of milk proteins
mRNA levels (%vs C)
250
200
150
100
50
0
C
Leu
*
CSN1S1
CSN1S2A
CSN2
CSN3
Amino acid transport
mRNA levels (%vs C)
150
100
50
0
C
Leu
ASC
LAT1
CAT1
rB16a

Figure 3

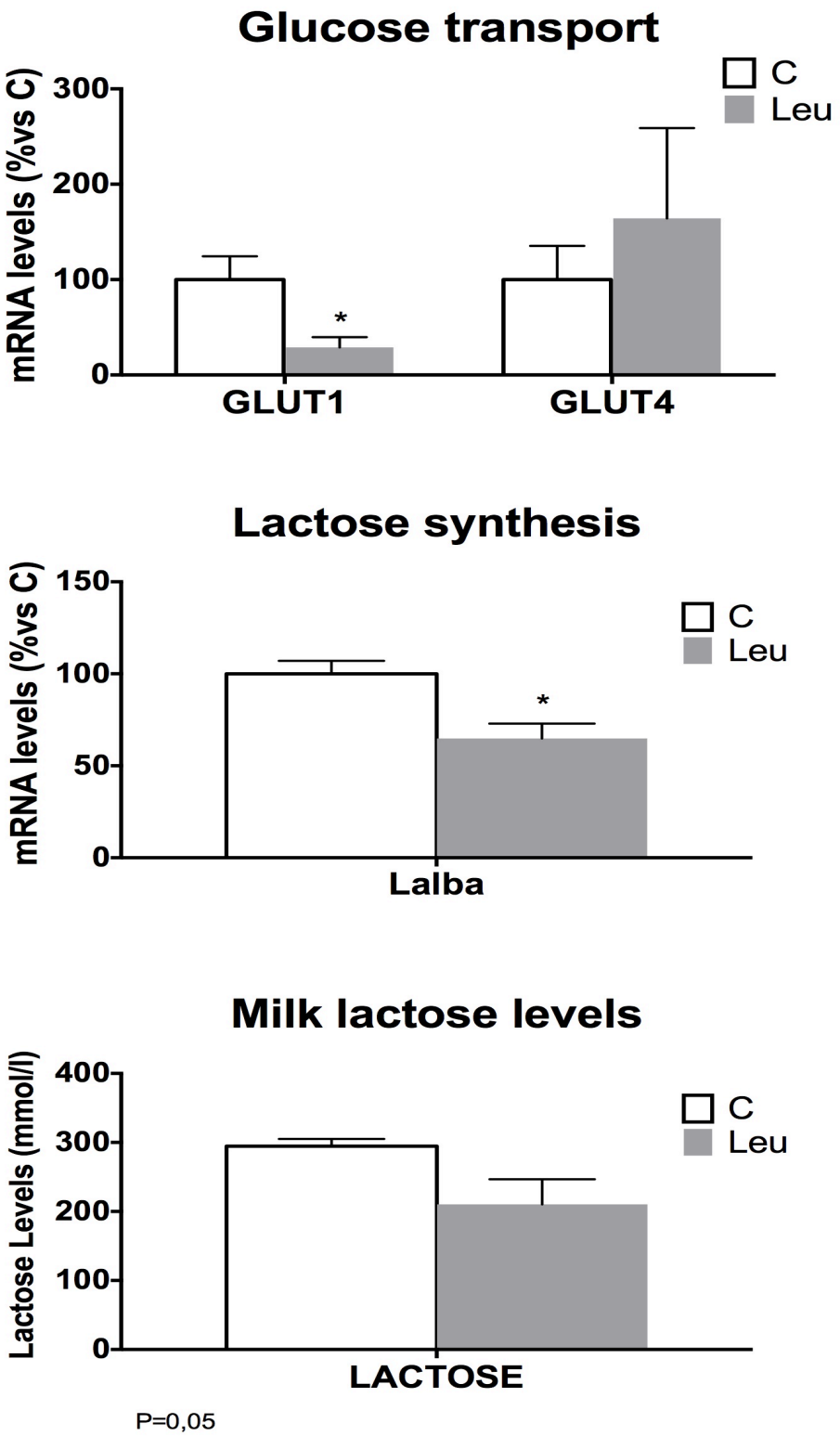

Glucose transport
mRNA levels (%vs C)
300
200
100
0
C
Leu
*
GLUT1
GLUT4
Lactose synthesis
mRNA levels (%vs C)
150
100
50
0
C
Leu
*
Lalba
Milk lactose levels
Lactose Levels (mmol/l)
400
300
200
100
0
C
Leu
LACTOSE
P=0,05

Figure 4

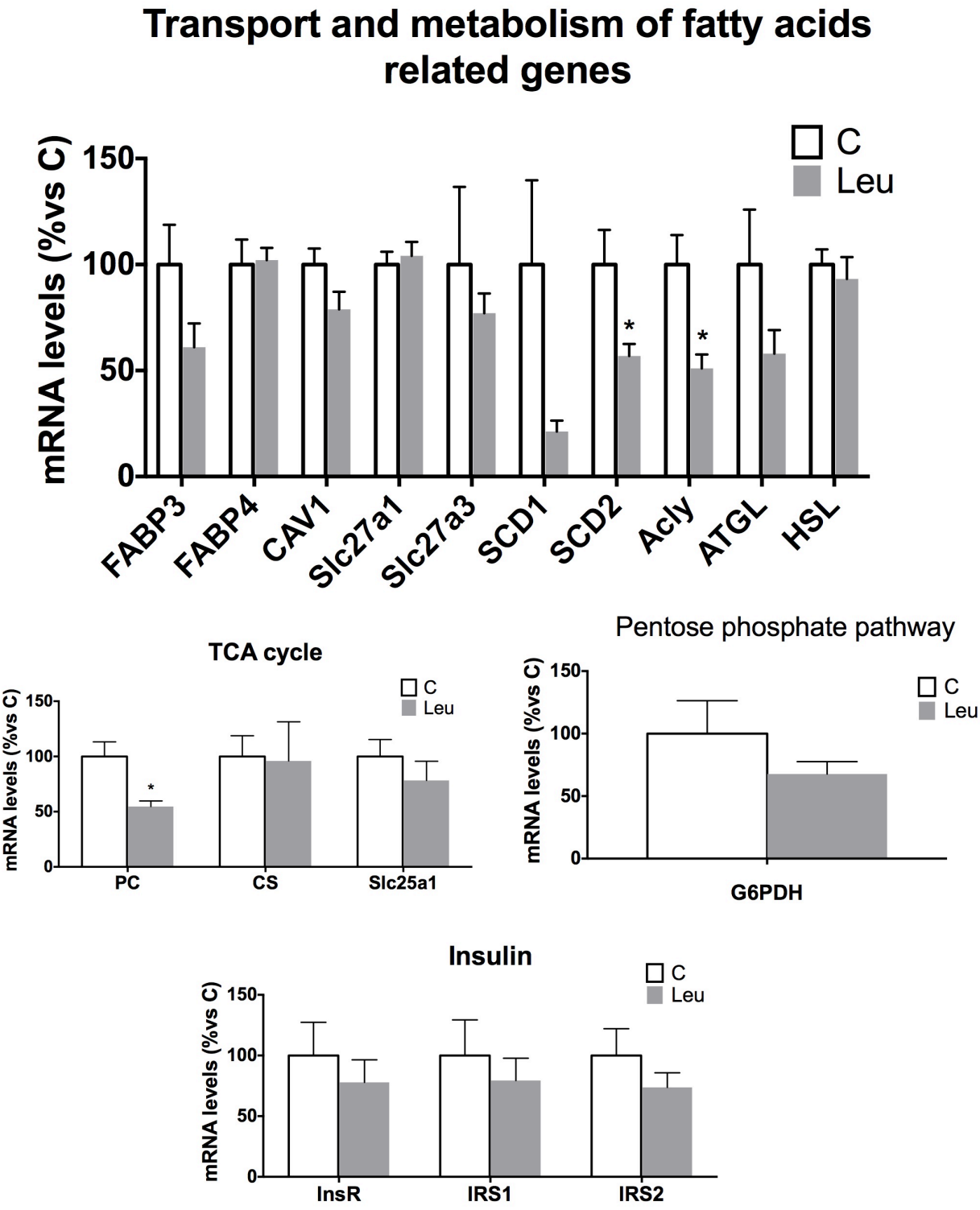
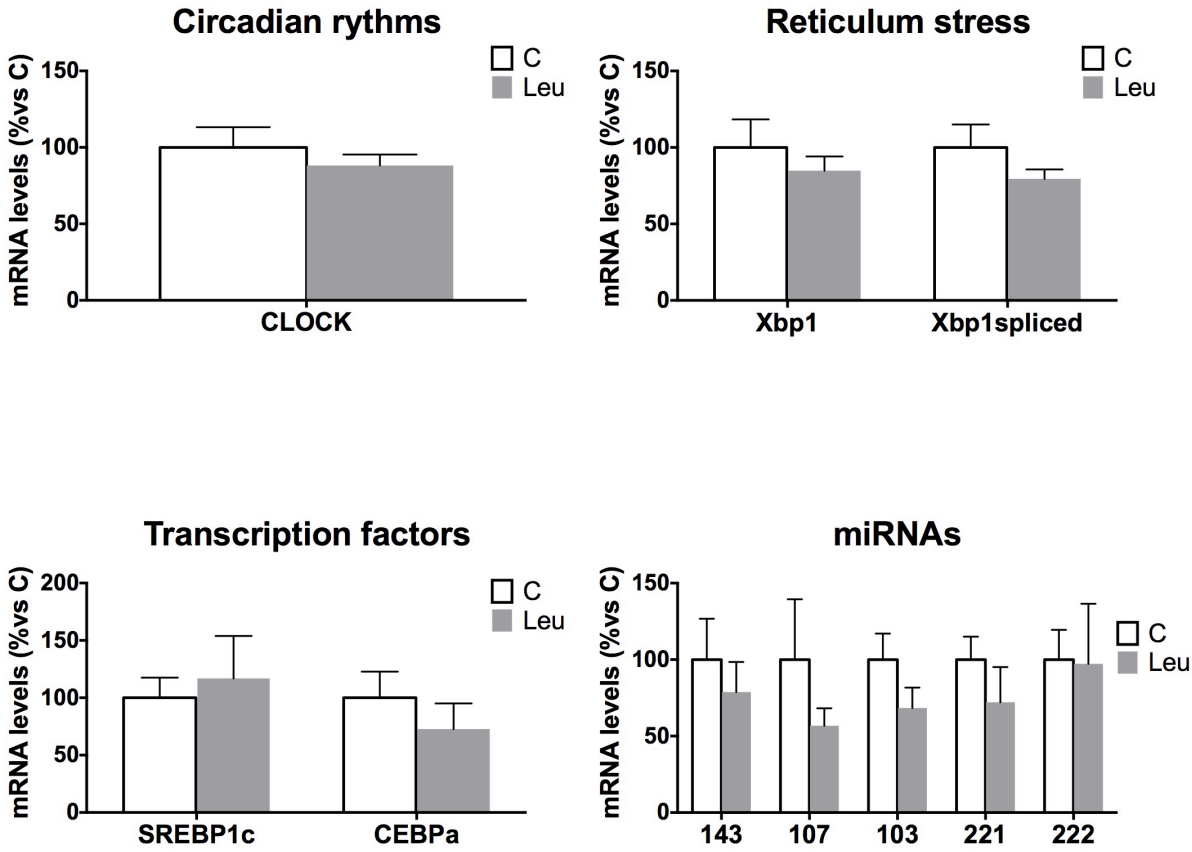

Transport and metabolism of fatty acids related genes
mRNA levels (%vs C)
150
100
50
0
C
Leu
*
*
FABP3
FABP4
CAV1
Slc27a1
Slc27a3
SCD1
SCD2
Acly
ATGL
HSL
TCA cycle
mRNA levels (%vs C)
150
100
50
0
C
Leu
*
PC
CS
Slc25a1
Pentose phosphate pathway
mRNA levels (%vs C)
150
100
50
0
C
Leu
G6PDH
Insulin
mRNA levels (%vs C)
150
100
50
0
C
Leu
InsR
IRS1
IRS2

Figure 5


Circadian rythms
mRNA levels (%vs C)
150
100
50
0
C
Leu
CLOCK
Reticulum stress
mRNA levels (%vs C)
150
100
50
0
C
Leu
Xbp1
Xbp1spliced
Transcription factors
mRNA levels (%vs C)
200
150
100
50
0
C
Leu
SREBP1c
CEBPa
miRNAs
mRNA levels (%vs C)
150
100
50
0
C
Leu
143
107
103
221
222

MANUSCRITO IV

Expression of brite adipocyte biomarkers show gender differences and the influence of early dietary exposure.

Servera M, López N, Serra F, Palou A.

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Abstract:	Induction of brown-like adipocytes (brite) in white adipose tissues may allow the conversion of lipid storage cells in fat burning cells. Little is known concerning browning potential in males compared with females. In this study, we aimed to analyse whether gender differences were present in gene expression of "brite" markers as well as the impact of dietary manipulation at both, early stages and adulthood in rats. We have determined the expression of brite markers and genes associated with lipid and energy metabolism in inguinal adipose tissue in adult male and female rats. We have analysed the impact of high-fat (HF) diet in adult life and of early leucine supplementation (2%) during lactation. Results show that although both genders have the potential to induce brite genes in inguinal adipose tissue, males expressed higher levels (Cidea, Hoxc9 and Shox2), which would imply a higher browning capacity in comparison with females. Minor impact of HF diet in adult life was observed in most of the genes studied. Interestingly, results showed that early Leu was able to compromise the metabolic fate of white and brite adipocytes later in adult life. Leucine supplementation programmed higher expression of CIDEA, accompanied with induction of SREBP and lower UPC2 expression, particularly in females. In addition, Leucine supplementation was associated with higher expression of leptin and PPAR and decreased CPT1 in both genders. Although the exact role of these adaptations needs further comprehensive analysis, dietary Leu supplementation at early age programmed inguinal adipose tissue in a gender specific manner.
Suggested Reviewers:	Stephan Herzig, Prof. Dr. Division Head, German Cancer Research Center s.herzig@dkfz.de He is leading the project DIABAT, which is focused on the role of brite cells as potential tools to combact obesity and diabetes type 2.
	María Cinta Bladé, PhD.

	Prof. Dr., University Rovira i Virgili mariacinta.blade@urv.cat She is a very active scientist looking for bioactive compounds that may activate metabolic pathways contributing to counteract obesity
	Jong W Yun, Dr. Daegu University jwyun@daegu.ac.kr He has characterized gender dymorphism in adipose tissue at proteomic level.
Opposed Reviewers:	

Expression of brite adipocyte biomarkers show gender differences and the influence of early dietary exposure

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Abstract

Induction of brown-like adipocytes (brite) in white adipose tissues may allow the conversion of lipid storage cells in fat burning cells. Little is known concerning browning potential in males compared with females. In this study, we aimed to analyse whether gender differences were present in gene expression of "brite" markers as well as the impact of dietary manipulation at both, early stages and adulthood in rats. We have determined the expression of brite markers and genes associated with lipid and energy metabolism in inguinal adipose tissue in adult male and female rats. We have analysed the impact of high-fat (HF) diet in adult life and of early leucine supplementation (2%) during lactation.

Results show that although both genders have the potential to induce brite genes in inguinal adipose tissue, males expressed higher levels (Cidea, Hoxc9 and Shox2), which would imply a higher browning capacity in comparison with females. Minor impact of HF diet in adult life was observed in most of the genes studied. Interestingly, results showed that early Leu was able to compromise the metabolic fate of white and brite adipocytes later in adult life. Leucine supplementation programmed higher expression of CIDEA, accompanied with induction of SREBP and lower UPC2 expression, particularly in females. In addition, Leucine supplementation was associated with higher expression of leptin and PPARγ and decreased CPT1 in both genders. Although the exact role of these adaptations needs further comprehensive analysis, dietary Leu supplementation at early age programmed inguinal adipose tissue in a gender specific manner.

Introduction

Adipose tissue of mammals is integrated by at least two types of adipocytes, white and brown, which are organised in white (WAT) and brown (BAT) adipose tissues constituting a multidepot organ called the adipose organ (Cinti, 2005). This is a major endocrine organ exerting a profound influence on whole-body homoeostasis and body weight control (Saely et al., 2012). Both type of adipocytes share the property of accumulation and release of fatty acids and they both express the rather specific adrenergic receptor β3 (Richard and Picard, 2011). However, they have distinct anatomy and function: white adipocytes are unilocular and regulate energy balance through secretion of leptin, while brown adipocytes are multilocular and rich in mitochondria and contribute to regulation of energy expenditure by means of UCP1 protein, responsible for the heat production of these cells (Cinti, 2005).

WAT and BAT are normally localized in anatomically distinct areas, although the presence of brown adipocytes expressing UCP1 in typical white fat pads also occurs under certain conditions (Cousin et al., 1992; Virtanen et al., 2009). Recently, molecular characterization of WAT has revealed the possibility of inducing a "browning process" in response to appropriate stimuli, like activation of β-adrenergic receptor (Ghorbani and Himms-Hagen, 1997), long term exposure to cold (Barbatelli et al., 2010), drugs (Distel et al., 2012) or even nutritional agents (Bonet et al., 2013). The mechanism of this plasticity is yet unknown and is under extensive investigation. These novel thermogenic brown-like adipocytes are also called "brite" (brown-in-white) cells, they are energy-expending cells found among energy-storing adipocytes and share characteristics with classical brown adipocytes (like the capacity for uncoupled respiration), are developed in the postnatal period and are susceptible of activation by cold exposure or PPARγ agonists (Sharp et al., 2012).

In adult humans, novel imaging techniques have demonstrated the existence of active brown adipose tissue, which is acutely induced by cold and stimulated via the sympathetic nervous system (Nedergaard et al., 2007; Saito, 2013). Furthermore, patients affected by pheochromocytoma secrete huge amounts of catecholamines and show high levels of active BAT by adrenergic stimulation (Frontini et al., 2013). Feasibility of browning process either by transdifferentiation (white adipocytes converted to brown) and/or by induction of novel brown/brite adipocytes in humans may provide an important strategy in obesity treatment, as these cells may contribute to attenuate the tendency to increase fat deposition seen in mice (Lasar et al., 2013).

Sex differences associated with fat distribution and correlations to metabolic health in humans are well established. Studies on morphological and metabolic properties of adipocytes, as well as on proliferation and differentiation potential of pre-adipocytes derived from different depots, with the purpose of understanding the intrinsic gender-specific metabolism have been performed (Karastergiou et al., 2012). However, little is known concerning gender differences in the metabolism of brite adipocytes and the changes occurring at gene expression level in the browning process in males and females. In this study, we aimed to analyse whether gender differences were present in gene expression of "brite" markers as well as the impact of dietary manipulation at both early stage (maternal supplementation with leucine) and adulthood (high-fat diet) in rats.

Materials and methods

Experimental animals

Animals were submitted to two dietary treatments: maternal leucine supplementation during lactation (L) and HF diet at adulhood (HF). L group was obtained by feeding powder standard diet (1.1% Leu) supplemented with 2% L-Leucine (>99% NT, Sigma-Aldrich, Madrid,

Spain) from day 1 after delivery until weaning at 21 days of age (López et al., 2010). Control animals (C) were treated in the same conditions but fed with the non supplemented powder standard diet. Then, offspring were caged by gender-mated animals and fed with standard diet. At the age of 6 months, both L and C animals were distributed into two dietary regimes, receiving either a high carbohydrate (HC) (3.85 kcal/g, 10% calories from fat, Ref D12450B) or a high fat (HF) diet (4.73 kcal/g, 45% calories from fat, Ref D12451B) (Research Diets (NJ, USA)). A total of 8 experimental groups were followed (n=6 each) according to diet (HC or HF), early treatment (C or L) and sex (males, females). At the age of 9 months (275 days), animals were sacrificed and adipose samples collected. The inguinal depot was rapidly removed, weighed, frozen in liquid nitrogen and stored at -80ºC. Diagram of the experimental dietary design is shown in Fig 1.

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

RNA extraction

Total RNA was extracted from the inguinal adipose depot by Tripure Reagent (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions. Final RNA precipitation was performed with 3M sodium acetate. Isolated RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and its integrity confirmed by agarose gel electrophoresis.

Real-time quantitative polymerase chain reaction analysis

Real-time polymerase chain reaction (PCR) was used to measure mRNA expression levels of selected markers of brite, brown and white adipocytes. Guanosine diphosphate

dissociation inhibitor (GDI) was used as reference gene. 0.08 μg of total RNA (in a final volume of 5 μl) was denatured at 90ºC for 1 min and then reverse transcribed to cDNA using MuLV reverse transciptase (Applied Biosystem, Madrid, Spain) at 42ºC for 60 min and 99ºC for 5 min in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain). Each PCR was performed in diluted (1/5) cDNA template, forward and reverse primers (10μM each) and Power SYBER Green PCR Master Mix (Applied Biosystem, CA, USA). Primers were obtained from Sigma Genosys (Sigma Aldrich Química, Madrid, Spain) and sequences are described in Table 1. Real-time PCR was performed using StepOnePlus Real-Time PCR System (Applied Biosystems) with the following profile: 10 min at 95ºC, followed by a total of 40 two-temperature cycles (15s at 95ºC and 1 min at 60ºC). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.2), and the relative expression of each mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) corrected by the expression of GDI in the sample. Data are presented as percentage of the values found in male control rats under HC diet (C-HC).

Statistical analysis

All data are expressed as the mean ± S.E.M. Two-way ANOVA was used to assess differences by early treatment (C *vs* L) and diet in adulthood (HF *vs* CH) in males and females. Three-way ANOVA was used to determine the existence of effects of gender (S), treatment (T) and diet (D). The analyses were performed with SPSS for Windows (SPSS, Chicago, IL). Threshold of significance was set at $P<0.05$.

Results

Males show higher levels of expression of brite markers than females in inguinal adipose tissue

Expression of markers associated with brown/brite adipocytes has been found in inguinal adipose tissue in both adult male and females. Interestingly, different expression pattern, mainly related to gender was observed within the biomarkers selected (Figure 2). Minor effects of early dietary treatment and diet composition in adult life were shown.

Cell death-inducing DFFA-like effector (CIDEA) is a structural brown adipocyte gene also present in brite cells. CIDEA expression was higher in males than females (S, p=0.000) and a dietary effect (D, p=0.031) was also observed, mainly associated with higher expression in HF-fed animals. Furthermore, Leu-treated females showed higher CIDEA values in comparison with the respective control groups (T, p=0.019). T-box15 (Tbx15) is another established gene as a brown/brite adipocyte marker. Tbx15 was detected in both males and females; however, its high variability in expression indicated that it was not sensible to the variables analysed. Uncoupling protein 1 (UCP1), the canonical marker of BAT, could not be detected in a consistent manner in all samples (data not shown) and the same was seen concerning messenger for PR domain containing 16 (PRDM16) a transcriptional coregulator that controls the development of classical brown adipocytes.

Homeobox C9 (Hoxc9) and short stature homeobox 2 (Shox2) genes have been characterized as brite adipocytes gene markers. Concerning their expression, no significant differences were shown associated either to diet or treatment. However, both biomarkers showed higher levels of expression in males than in females under all conditions studied (S, p=0.000 and p=0.001, respectively).

Males show higher levels of expression of genes than females in inguinal adipose tissue

We have further characterised inguinal gene expression in order to get insight into the metabolic adaptations and potential influence of brite cells in the inguinal depot. Most of the

genes showed a clear gender influence, presenting higher expression in males than females as observed with brown/brite biomarkers. Expression pattern of a few genes could be associated with early dietary treatment and only fatty acid synthase (Fasn) to current dietary feeding (Figure 3).

Gene expression of regulatory proteins secreted by adipocytes like leptin (Lep), resistin (Retn) and adiponectin (AdipoQ) were higher in males than in females (S, p=0.000, p=0.000 and p=0.002 respectively). In addition, influence of early Leu-treatment (T, p=0.006) was associated with higher expression of leptin in females.

Managing of triglycerides/fatty acids by adipose triglyceride lipase (ATGL) and lipoprotein lipase (LPL) was also higher in males than females (S, p=0.001 and p=0.0000, respectively), and this pattern was also found in the expression of other genes dealing with fatty acid synthesis and handling. Carnitine palmitoyl transferase (CPT1m), Fasn and stearoyl-CoA desaturase 2 (SCD2) showed higher levels in males than in females (S, p=0.049, p=0.000 and p=0.012, respectively). In addition, Fasn expression was decreased in high-fat fed animals (D, p=0.047) and CPT1m in Leu-supplemented animals (T, p=0.022). Transcription factors associated with lipid metabolism such as sterol regulatory element binding transcription factor (SREBP1c) and peroxisome proliferator activated receptor gamma (PPARγ2) were also higher in males than in females (S, p=0.000 and p=0.000, respectively) and reflected the impact of early dietary treatment; PPARγ2 expression was higher in Leu-treated animals (T, p=0.006) and SREBP1c in Leu-treated females (T, p=0.045). Regarding the uncoupling protein, expression of UCP2 was affected by early Leu-treatment which was decreased in females (T, p=0.019), particularly under the HC diet.

Capacity to transport glucose mediated by Glut4 showed the same tendency as seen for fatty acid-related genes, being higher in males than in females (S, p=0.002) and it was not influenced by early Leu-treatment or diet composition in adult life.

The marker of macrophage infiltration (CD36) was also higher in males than females (S, p=0.000) in accordance with the rest of the genes analysed. Expression of X-box binding protein 1 (XBP1), part of the endoplasmic reticulum (ER) stress response, was not different between groups.

Discussion

It is known that gender differences in obesity originate mainly from hormonal and metabolic differences between sexes. Recently, a series of proteomic analysis have revealed some of the gender-specific characteristics of the proteome profile in BAT (Choi et al., 2011) and WAT depots (Mukherjee et al., 2012). Gender dimorphism is present at the level of adipose protein expression and affects a reduced number of proteins (between 25 and 46 depending on the source of the depot) (Mukherjee et al., 2012). In accordance, previous data from our lab also show lower transcriptomic expression in females compared to male rats. Interestingly, differences between sexes are more marked in inguinal WAT (Priego et al., 2008). Subcutaneous white adipose depots have a higher propensity toward expression of brite markers compared to the visceral depots (Wu et al., 2012). However, no data are available concerning gender differences in the potential of the inguinal adipose tissue to express brite-associated genes. Our data show that although both genders express and therefore may induce, brite genes in inguinal adipose tissue, males expressed higher levels of Cidea, Hoxc9 and Shox2 and the same trend was detected for Tbx15 (Figure 2). This pattern was a reflection of metabolic potential of the tissue in a gender-related manner. Therefore, in comparison with females, males presented higher expression of adipocyte-associated regulatory proteins such as leptin, adiponectin and resistin; higher handling capacity of lipids/fatty acids by LPL and ATGL, and their processing by Fasn, CPT1m and SCD2. In addition, lipid regulatory factors such as SREBP1c and PPARγ2 were also expressed at higher level in males than in females (Figure 3). All together, the data indicate that female rats have lower lipid metabolic capacity in inguinal

adipose tissue in comparison with males. This pattern, shared by brite-associated genes too, may suggest a lower browning capacity in white adipose tissue of female rats in comparison with males.

Another question raised in the present study was the relevance of dietary impact in the expression of brite genes in a gender specific manner. We were interested in analysing the impact of diet composition in adult life but also in identifying effects of early dietary manipulation on brite expression in rats. Diets with a high content of fat (HF) or carbohydrate (HC) were selected in adult life, as tools to check the potential of brite induction in inguinal adipose. No data are available on the performance of inguinal WAT on brite induction by dietary manipulation in rats. Most of the studies have been performed in mice and the general view is that HF feeding may increase thermogenesis in BAT but reduces the brite potential in WAT. In this context, brite adipocytes would be displaced by white adipocytes to store fat in a time of surplus (Bonet et al., 2013). However, differences between species may occur as increased expression of UCP1 mRNA by HF feeding has been found in abdominal fat in female rats (Margareto et al., 2001) and in retroperitoneal adipose tissue in male hypertensive rats (Hojna et al., 2012). We were unable to detect UCP1 expression and minor impact of HF diet in adult life was observed in the rest of the genes studies. CIDEA was the only brite-gene affected by the dietary treatment, showing increased expression in HF fed animals compared with the HC diet, a pattern shared by both genders. The role of CIDEA has been associated with lipid droplet enlargement and appears broadly expressed in most of the multilocular cells. Furthermore, the presence of CIDEA positive with UCP1 negative multilocular adipocytes in WAT depots in cold acclimated mice has been proposed as an early stage on adipocyte transdifferentiation (Barneda et al., 2013). Although this is a possibility that cannot be ruled out in our experimental setup, it would need further investigation.

Interestingly, it is shown that early dietary manipulation is able to compromise the metabolic fate of white and brite adipocytes later in adult life. We have used a model of

maternal dietary leucine supplementation during lactation (López et al., 2010). In this model, nursing dams show a healthier profile of body composition, without compromising the growth and development of the progeny by a mechanism that would involve lower expression of orexigenic neuropeptides in hypothalamus (López et al., 2010). Here we show that early leucine supplementation programmed higher expression of CIDEA, which could be regulated by the induction of SREBP and was accompanied by lower UPC2 expression, particularly in females. In addition, early leucine supplementation was associated with higher expression of leptin and PPARγ together with decreased CPT1 in both genders. In adipocytes, CIDEA seems to mediate SREBP-dependent lipid accumulation under the control of PPARγ (Puri et al., 2008). Our results fit with this model and altogether seem to indicate that the main effect of early leucine supplementation was the promotion of lipid storage, particularly in female offspring. Although the exact role of these adaptations needs further comprehensive analysis, dietary leucine supplementation at early age programmed inguinal adipose tissue in a gender specific manner.

Taken together, this study demonstrated that the level of expression of genes related to browning process in adipose tissue is gender dependent, male rats presenting higher levels than females. This pattern is similar to that found for most of genes associated with adipocyte metabolism and analysed in the present study. Furthermore, our data show minor impact of diet composition in adult life in the expression pattern of brite biomarkers, whereas influence of early dietary supplementation to maternal diet seems to have higher relevance. This knowledge may provide bases for specific evidence-based interventions and therefore allowing for more focused prevention and/or treatment of obesity.

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Conflict of interest

The authors declare that they have no conflict of interest. Authors declare that they have full control of all primary data and agree to allow the journal to review their data if requested. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Legends to table and figures

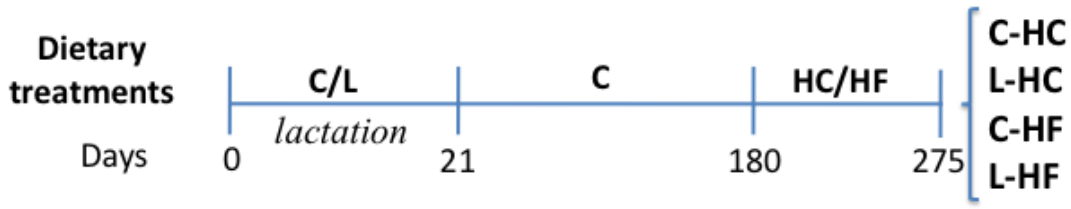
Table 1. Nucleotide sequences of primers

Figure 1. Diagram representing the experimental design and diets. C, fed with chow diet; L, chow diet supplemented with 2% L-Leucine; HC, high carbohydrate diet and HF, high fat diet.

Figure 2. mRNA levels in the inguinal adipose tissue of 9-month-old rats (males and females) fed with a high fat (HF) or high carbohydrate (HC) diet. L and C make reference to early dietary treatment that was either leucine (L) or control (C); mRNA levels were measured by real-time PCR and expressed as a percentage of the value of male control animals under HC diet (C-HC group). Results are mean ± S.E.M (n=6). Effect of sex (S), diet (D) or treatment (T) was assessed by two/three way ANOVA (significance was set at $p<0,05$). Cell death-inducing DFFA-like effector (CIDEA), T-box 15 (TBX15), Homeobox C9 (HOXC9), Short stature homeobox 2 (SHOX2).

Fig. 3 mRNA levels in the inguinal adipose tissue of 9-month-old rats (males and females) fed with a high fat (HF) or high carbohydrate (HC) diet. L and C make reference to early dietary treatment that was either leucine (L) or control (C); mRNA levels were measured by real-time PCR and expressed as a percentage of the value of male control animals under HC diet (C-HC group). Results are mean ± S.E.M (n=6). Effect of sex (S), diet (D) or treatment (T) was assessed by two/three way ANOVA (significance was set at $p<0,05$). Cluster of differentiation 36 (CD36), Carnitine palmitoyltransferase 1b muscle isoform (CPT1m), fatty acid synthase (FASN), facilitated glucose transporter 4 (GLUT4), leptin (LEP), lipoprotein lipase (LPL), peroxisome proliferator activated receptor gamma 2 (PPARγ2), sterol regulatory element binding transcription factor 1c (SREBP1c), stearoyl-Coenzyme A desaturase2 (SCD2), uncoupling protein 2 (UCP2), adiponectin (ADIPOQ), X-box binding protein 1 (XBP1), adipose triglyceride lipase (ATGL), resistin (RETN).

Figure 1


Dietary treatments
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C
HC/HF
Days
0
21
180
275
C-HC
L-HC
C-HF
L-HF

Figure 2

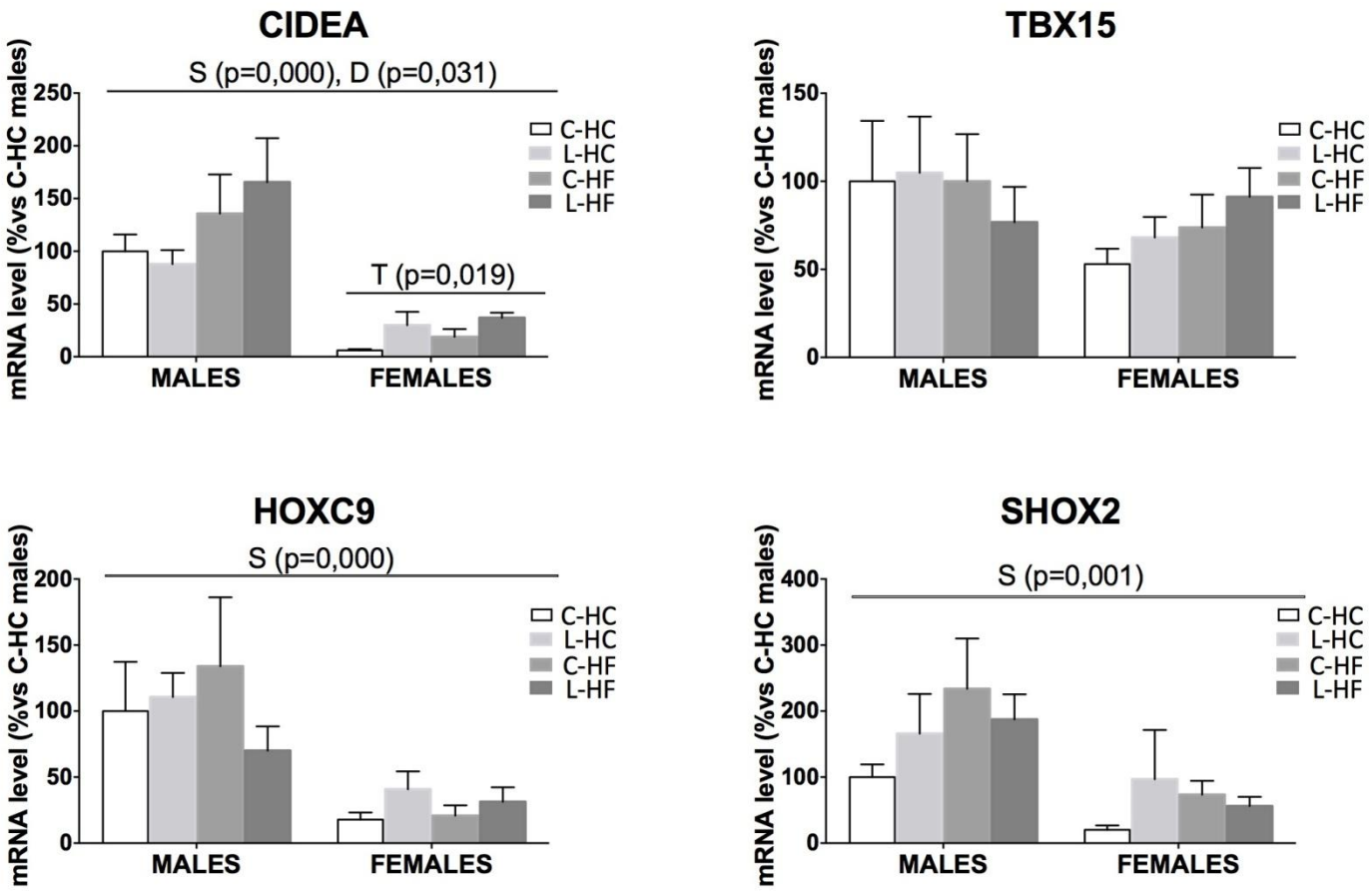
CIDEA
S (p=0,000), D (p=0,031)
T (p=0,019)
mRNA level (%vs C-HC males)
250
200
150
100
50
0
MALES
FEMALES
C-HC
L-HC
C-HF
L-HF
TBX15
150
100
50
0
HOXC9
S (p=0,000)
SHOX2
S (p=0,001)
400
300

Figure 3

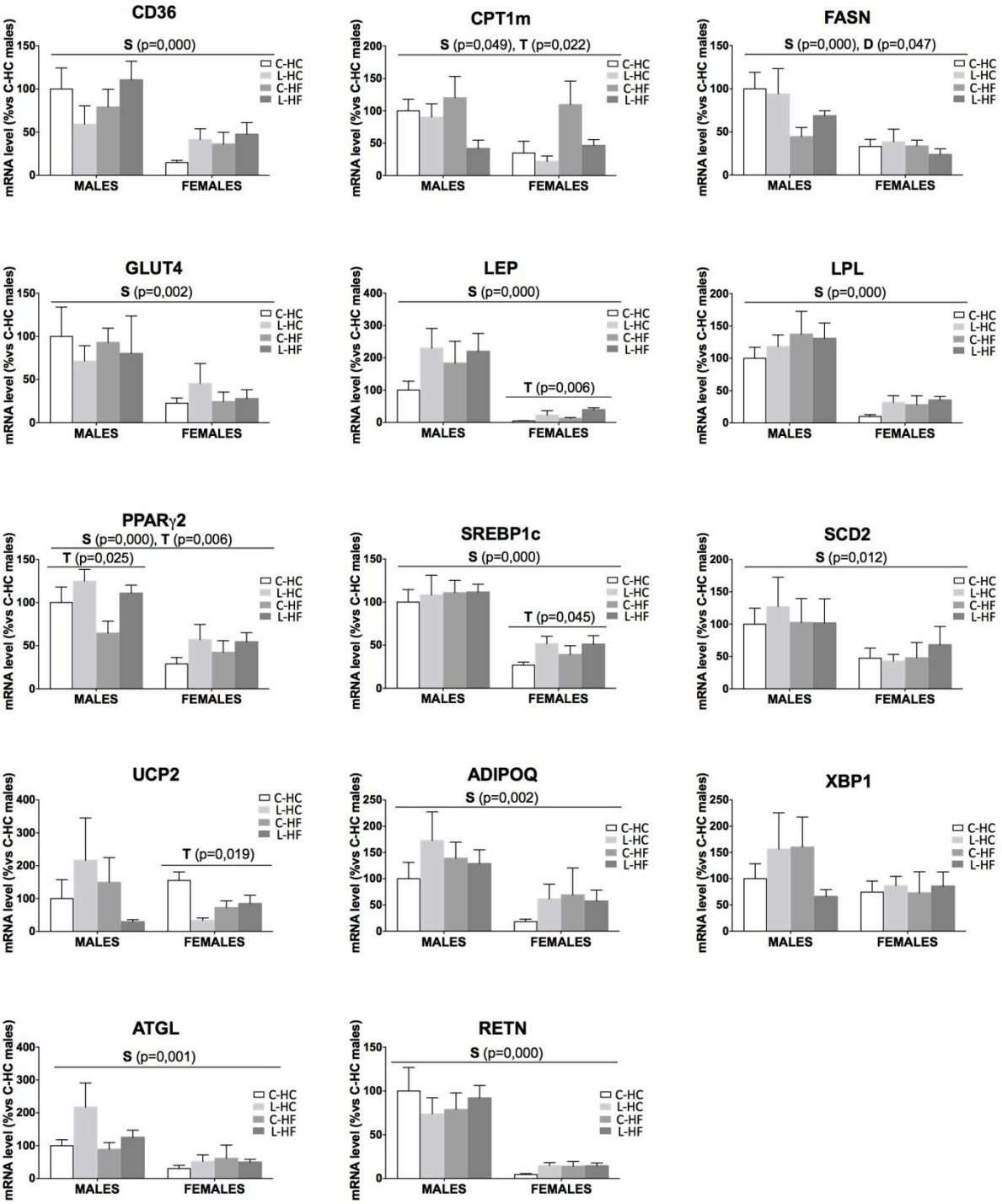
CD36
S (p=0,000)
CPT1m
S (p=0,049), T (p=0,022)
FASN
S (p=0,000), D (p=0,047)
GLUT4
S (p=0,002)
LEP
S (p=0,000)
T (p=0,006)
LPL
S (p=0,000)
PPARγ2
S (p=0,000), T (p=0,006)
T (p=0,025)
SREBP1c
S (p=0,000)
T (p=0,045)
SCD2
S (p=0,012)
UCP2
T (p=0,019)
ADIPOQ
S (p=0,002)
XBP1
ATGL
S (p=0,001)
RETN
S (p=0,000)
mRNA level (%vs C-HC males)
C-HC
L-HC
C-HF
L-HF
MALES
FEMALES

Table 1

Gene [a]	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon size (bp)
Adipoq	GCTCAGGATGCTACTGTTG	TCTCACCCTTAGGACCAAG	241
ATGL	TGTGGCCTCATTCCTCCTAC	AGCCCTGTTTGCACATCTCT	271
CD36	GTCCTGGCTGTGTTTGGA	GCTCAAAGATGGCTCCATTG	319
CIDEA	TCAGACCCTAAGAGACAACACA	CATTGAGACAGCCGAGGA	164
CPT1	GCAAACTGGACCGAGAAGAG	CCTTGAAGAAGCGACCTTTG	180
FASN	CGGCGAGTCTATGCCACTAT	ACACAGGGACCGAGTAAT	222
GDI	CCGCACAAGGCAAATACATC	GACTCTCTGAACCGTCATCAA	159
GLUT4	GGCATGCGTTTCCAGTATGT	GCCCCTCAGTCATTCTCATC	233
HOXC9	CGGCAGCAAGCACAAAGA	AGAAACTCCTTCTCCAGTTCCA	138
LEPTIN	TTCACACACGCAGTCGGTAT	AGGTCTCGCAGGTTCTCCAG	186
LPL	TATGGCACAGTGGCTGAAAG	CTGACCAGCGGAAGTAGGAG	157
PPARγ2	GATCCTCCTGTTGACCCAGA	TCAAAGGAATGGGAGTGGTC	164
RETN	CTCCTCCTTTTCCTTTTCTTCC	TAGTGACGGTTGTGCCTTCT	205
SCD2	TTTCTCATCATCGCCAACAC	CTTTCCGCCCTTCTCTTTG	189
SHOX2	GCTGACGGAGGTGTCCCCTGA	CGAAAAGCCTCTCCAGCTCGTT	143
SREBP1c	CCCACCCCCTTACACACC	GCCTGCGGTCTTCATTGT	198
TBX15	GGATGAGACAGGTGGTCAGTT	CACAGGCACAGGTT	164
UCP2	GGTCGGAGATACCAGAGCAC	ATGAGGTTGGCTTTCAGGAG	174
XBP1	TCCGCAGCACTCAGACTATGT	ATGCCCAAAAGGATATCAGACTC	129

[a] ADIPOQ, adiponectin; ATGL, adipose triglyceride lipase; CD36, cluster of differentiation 36; CIDEA, cell death-inducing DFFA-Like Effector A; CPT1, carnitine palmitoyltransferase;

FASN, fatty acid synthase; GDI, guanosine diphosphate dissociation inhibitor; GLUT4, facilitated glucose transporter 4; HOXC9, homeobox C9; LPL, lipoprotein lipase; PPARγ2, peroxisome proliferator activated receptor gamma 2; RETN, resistin; SCD2, stearoyl-Coenzyme A desaturase2; SHOX2, short stature homeobox 2; SREBP1c, sterol regulatory element binding transcription factor 1c; TBX15, t-box 15; UCP2, uncoupling protein 2; XBP, X-box binding protein.

---------- Original Message ----------
From: Fabio Virgili <virgili@inran.it>
To: francisca serra <francisca.serra@uib.es>
Date: 12 / juny / 2013 at 17:14
Subject: GENU: Your manuscript entitled Expression of brite adipocyte biomarkers show gender differences and the influence of early dietary exposure
Ref.: Ms. No. GENU-D-13-00039
Expression of brite adipocyte biomarkers show gender differences and the influence of early dietary exposure
Genes & Nutrition

Dear Prof. serra,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, the responsible editor would be glad to reconsider a revised version.

For your guidance, reviewers' comments are appended below.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript.

Your revision is due by 11-08-2013.

To submit a revision, go to http://genu.edmgr.com/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Santhi Natraj
JEO Assistant
Genes & Nutrition

Reviewers' comments:

Dear Dr Serra,
two expert reviewers have now provided their comments. They considered your ms of potential interest for the readers of G&N, but not in the present form. I therefore invite you to submit a revised version of your manuscript tacking into account all the points raised by the reviewers. Please note that submitting a revised manuscript does not warrant that your paper will be automatically accepted.
Thanking you for your interest in publishing your research in G&N,
best regards
The Editor in chief
Fabio Virgili

Reviewer #1: The paper by Servara et al. aims to investigate potential gender differences in brite adipocytes on the level of gene regulation. I addition authors aimed to investigate a potential impact of dietary intervention both in an early stage of development and in adulthood. Authors investigated the expression of 18 genes totally, 15 of which were expressed to a considerable higher levels in males, the expression of three was unaffected by gender. None of the investigated genes was higher in females. This exclusive regulation is surprising. In addition dietary intervention with leucin during lactation and diet (high fat vs. high carbohydrate) in adulthood seems to mildly impact on regulation of gene expression of investigated "brite" genes. These findings basically merit publication in GENU, however manuscript needs considerable revision.

Major issues:

Normalization of PCR data: As all data are normalized to the data from the C-HC group in males, which are set to 100 %. This implies that the house keeping gene GDI is expressed to a similar level in males and in females, otherwise this comparison is not valid for gender differences. Authors should give the expression levels (Delta-ct values and not Delta-Delta-ct values) for the comparison of the expression of the house keeping gene in males and females.

Translation into protein levels: In their discussion authors refer to gender differences of adiopose protein expression. This is exactly the point which needs to be addressed. Using the protein with the most pronounced level of changes on the mRNA level (CIDEA) authors should also include a Western-blot ananlysis to proof that these gender differences also hold on the protein level.

Fig. 3: All figures should be arranged in alphabetical order, otherwise the reader needs to search too much for the respective graph.

Annotation of significance data: In figures it is really hard to follow statistical significance of data. Authors either label statistical significance with a bar over eight experimental groups or at least 4 groups if changes can only be allocated to one gender. Although several comparisons apparently are done only one p-value is given, which implies that this value is valid for all conditions. This is confusing. I would like to highlight this with a few examples:
CIDEA: Authors claim influence of diet (D) in both genders and treatment (T)in females. They give a p-value of p=0,031 for D. Does this statistical significance hold for C-HC vs. C-HF and L-HC vs. L-HF each in both genders and with the same p-value? Likewise in females it is surprising that C-HC vs. L-HC and C-HF vs. L-HF should have the same level of significance.
Leptin: The same considerations hold for the influence of T again identical p-value
FASN: As shown for this gene, D influences in both genders with the identical p-value. This is unlikely
CTP1: The Leu effect appeara apparently only in HF
Generally: The annotation of statistical significance should be carefully revised for all analysed genes not only for those mentioned as an example

Discussion: Authors should explain the peak in C-HF for CTP1 females first, before they discuss results for this gene.

Discussion: CPT1, I can see the decrease in response to Leucin only in animals in HF

Discussion: As gender differences are apparent, it would be interesting to discuss the potential regulation of expression of the investigated genes through sex steroid hormone receptors. In other words, what is known about the regulation of the investigated genes through estrogens and androgens and which estrogen receptor is potentially involved.

Minor comments:

There are some minor typing errors

Reviewer #2: In this manuscript, the authors investigate about gender dimorphism in the expression of genes of adipose tissue. Experimental design is technically sound and some interesting results were found. However, some issues should be clarified as noted below.

Major concerns
1. The authors defined genes differentially expressed between gender and diet as "biomarkers". However, all 18 genes cannot be "biomarkers" to specify brite. Only in case those genes are regulated with the same patterns with their product proteins, those genes may be called as biomarkers. In this study, the authors should have presented protein levels of key player proteins that specify brite phenotype.

2. In the title, "brite" is not widely accepted term, thus title should be changed.

3. Although UCP1 is the most important genes in this study, data was not shown. Protein levelos of UCP1 should be prsented.

4. Data interpretation is not reasonable. For example, some genes showed significantly expressed between the gender, their physiological sigificances were not discussed.

V. Recapitulación

La lactancia materna tiene un importante papel sobre el crecimiento de la descendencia y constituye un momento crucial en su programación metabólica, modulando la propensión a determinadas enfermedades en edad adulta. La hipótesis central de desarrollo esta Tesis ha sido que la nutrición, particularmente en etapas tempranas del desarrollo, modula y/o condiciona la susceptibilidad a la obesidad en edad adulta y con ello condiciona la expresión génica en tejidos clave. El objetivo de la presente Tesis ha sido profundizar en el conocimiento de los efectos de dos nutrientes, la lactosa y la leucina, durante la lactancia. Se ha puesto interés en dos aspectos diferenciados, uno vinculado a la capacidad moduladora y secretora de la glándula mamaria, su plasticidad y papel en la definición de la composición de la leche; y el otro más centrado en la progenie, mediante la determinación del potencial "brite" en animales adultos, cuya caracterización frente a intervenciones nutricionales en diferentes periodos vitales aporta nuevas evidencias de la relevancia de la programación metabólica durante la lactancia.

I. TRANSFECCIÓN *IN VIVO* PARA LA OBTENCIÓN DE LECHE REDUCIDA EN LACTOSA

Reducción de lactosa en leche

La lactosa se sintetiza en la glándula mamaria por el complejo de la lactosa sintasa y constituye el principal azúcar presente en la leche (Jensen, 1995). Muchos adultos en todo el mundo muestran los síntomas asociados con la mala absorción de lactosa y son intolerantes a lactosa (Tunick, 2009; NDA, 2004). Tan es así que la lactosa ha sido incorporada en la lista de alérgenos de alimentos que tienen que ser incluidos obligatoriamente en el etiquetado de alimentos producidos bajo la normativa europea (véase el NDA, 2004).

Así pues, disminuir la concentración de lactosa en leche es un aspecto clave en el desarrollo de productos aptos para el consumo en el caso de intolerancia a la lactosa, que además permitiría mantener una ingesta de calcio adecuada y procedente de fuentes lácteas (las de mayor biodisponibilidad). De este modo, la fabricación de productos lácteos optimizados y específicos para consumidores intolerantes a lactosa es un área de innovación con importantes repercusiones económicas en el sector lácteo. Si bien,

aunque un informe reciente reconoce que los productos tratados con lactasa pueden ser mejor tolerados que los productos no tratados en las personas intolerantes a lactosa, también se pone de relieve que se requiere de más investigación específica en dicho tema (NIH, 2010).

Los primeros intentos de producir leche reducida en lactosa datan del siglo pasado. Así, en 1979 se desarrolló y comercializó leche conteniendo una enzima lactasa extraída de levadura y hongos (Tunick, 2009). Pero el interés sobre el tema sigue vigente, de modo que más recientemente ha sido evaluada positivamente por la Autoridad Europea de Seguridad Alimentaria una declaración de propiedades saludables asociada al consumo de la enzima lactasa y la hidrólisis asociada de la lactosa, para personas con síntomas de digestión deficiente de lactosa (NDA, 2009).

Existen varios métodos capaces de producir leche de bajo contenido en lactosa, incluyendo procedimientos in vitro que se aplican tras la recolección en las fases de procesamiento de la leche para su comercialización (Vilotte, 2002; Tamm, 1994;. Suárez et al, 1995) e in vivo mediante la reducción de la actividad del complejo enzimático responsable de la síntesis de lactosa; sin embargo, este último enfoque da lugar a una reducción global en el contenido de azúcar y resulta una leche altamente viscosa (Stinnakre et al, 1994; L'Huillier et al, 1996). La utilización de animales transgénicos que expresen en mayor medida la enzima responsable de hidrólisis de la lactosa (β-galactosidasa) o que expresen, en este caso en menor medida, la enzima responsable de la síntesis de lactosa a partir de UDP-galactosa y glucosa (complejo α-lactalbúmina) posibilita generar in vivo una leche "naturalmente" más adaptada para el consumo de los intolerantes a lactosa. Uno de los logros tempranos en este campo ha sido la obtención de ratones transgénicos que expresan la enzima que hidroliza la lactosa en la glándula mamaria in vivo (Jost et al., 1999). Sin embargo, la obtención de estos animales y su aplicación a mamíferos domésticos es en muchas ocasiones compleja y costosa, aparte de que las características organolépticas de la leche producida a veces tampoco son las deseadas (L'Huillier et al., 1996, Jost et al., 1999).

Desarrollo y aplicación de la transfección in vivo del gen β-galactosidasa

Nuestro planteamiento ha sido desarrollar y aplicar un método basado en la transfección in vivo en la glándula mamaria de ratas gestantes justo antes del parto. Se pretendía conseguir una leche de contenido reducido en lactosa in situ, que permitiera mantener el patrón de ingesta fisiológico de las crías lactantes y a la vez posibilitara la caracterización del impacto nutricional de la reducción de lactosa en leche materna con una mínima manipulación de los animales.

Para la transfección se ha utilizado un procedimiento basado en una solución de ADN / DEAE-dextrano, que se ha demostrado que funciona eficientemente en cobayas para obtener la expresión de la correspondiente proteína secretada en la leche (Hens et al., 2000) si bien hasta el momento, no había referencias de su aplicación en rata de laboratorio. De las dos alternativas principales existentes, sobre-expresar un enzima capaz de hidrolizar la lactosa o inhibir la expresión del enzima responsable de su síntesis, se seleccionó la utilización de la enzima β-galactosidasa recombinante. Es un enzima de amplio uso en múltiples aplicaciones industriales y biotecnológicas, la mayoría de ellas relativa a la eliminación de lactosa en productos lácteos (Husain, 2010; NDA, 2009). Asociado a ello, como vector de transferencia se eligió un plásmido comercial con capacidad demostrada en la expresión del DNA en células de mamífero. La primera utilización de este vector in vivo aparece en el 2009 y a día de hoy existen más descripciones de su uso (Ge et al., 2009, Forster et al., 2013) En definitiva, este planteamiento buscaba garantizar, dentro de un rango de posibilidades discreto, el éxito de la transferencia del material genético, su expresión localizada en glándula mamaria y centrarse en el impacto de la modificación de la composición en lactosa en las crías lactantes.

Los resultados obtenidos han mostrado la viabilidad de la transfección en glándula mamaria in vivo mediante la infusión de ADN plásmido con un gen que codifica la enzima recombinante β-galactosidasa. La expresión de la proteína heteróloga en leche ha permitido a las crías alimentarse con una leche reducida en lactosa y con mínima intervención humana. Por lo tanto ha posibilitado el estudio de los efectos metabólicos asociados a dicha modificación en la composición.

Es interesante destacar que la transfección previa al parto ha conseguido la síntesis de leche reducida en lactosa a lo largo del primer tercio de la lactancia, de modo

que se ha conseguido aunar el período de preparación de la glándula mamaria para hacer frente a la lactancia con el proceso de amplificación del plásmido inoculado. Por otra parte, el carácter transitorio del efecto deseado indica que no se ha producido la inserción del ADN plasmídico en el genoma materno, tal como se pretendía con el uso de esta técnica en comparación con la generación de transgénicos, si bien harían falta pruebas complementarias para demostrarlo.

El procedimiento aplicado ha logrado una disminución del 35% de los niveles de lactosa el día 6 de lactancia. La ausencia total de lactosa no era deseable, ya que, la lactosa, junto con otros azúcares e iones difusibles, es responsable de la presión osmótica de la leche (Stinnakre et al., 1994). Por lo tanto, el enfoque aplicado ha sido suficiente para producir una reducción significativa de la lactosa, por lo menos durante el primer tercio de la lactancia.

Consecuencias metabólicas asociadas a la producción in vivo de leche reducida en lactosa

El desarrollo y aplicación de la técnica de transfección ha sido un éxito ya que se ha conseguido la producción de leche con contenido reducido en lactosa en un rango fisiológico significativo, tanto a nivel temporal como de dosis. Si bien, asociado a ello se ha puesto en evidencia una serie de efectos metabólicos sobre las madres y también sobre las crías lactantes que abren nuevas expectativas inicialmente no anticipadas.

El análisis del peso y composición corporal de las madres lactantes ha mostrado que las que codifican para el enzima recombinante ß-galactosidasa presentaban una mayor masa grasa (39%) que los controles entre los 10 y 14 días después de la transfección. De acuerdo con ello, la leptina, que es la hormona responsable de informar al cerebro del estado nutricional y regular así la ingesta y el gasto energético (Cinti, 2005), también mostró niveles más altos en sangre en las madres tratadas con respecto a los controles. De modo que la inducción del enzima ß-galactosidasa en la glándula mamaria ha favorecido un balance energético positivo en el organismo materno sin modificar la ingesta y se produce una menor utilización de las reservas grasas maternas que permite mantener un peso superior al que presentan los animales no tratados.

Por otra parte, se esperaba que la hidrólisis de lactosa, asociada a la inducción de la enzima heteróloga, produjera glucosa y galactosa en leche en proporciones comparables a la disminución del contenido en lactosa. Sin embargo, la reducción de lactosa en leche ha ido acompañada de una reducción del contenido en galactosa (59% en el día 6), mientras que la concentración de la glucosa no se modifica a lo largo de la lactancia. El descenso en los niveles de galactosa y el mantenimiento de los de glucosa podrían estar ambos asociados a mecanismos de reabsorción de monosacáridos por las células alveolares de la glándula mamaria, como ocurre en el caso de ratones transgénicos que expresan lactasa intestinal en la leche (Jost et al., 1999); aunque no se ha abordado en esta tesis, estos resultados sugieren la presencia de mecanismos específicos, tal vez de índole absortiva, encaminados a la conservación de la concentración de glucosa. Lo que pone en evidencia el papel de la glándula mamaria en la modulación de la composición de la leche, en este caso para contrarrestar una intervención externa que afecta inicialmente a la concentración de lactosa.

Dado el potencial obesogénico de la intervención en el organismo materno, se quiso determinar el impacto del consumo de dicha leche en la descendencia adulta. Al igual que se ha observado en las madres, la progenie también presenta un mayor peso corporal, asociado con un aumento en la masa grasa, tanto en machos como en hembras, que no se puede relacionar con una mayor ingesta. Así pues, la alimentación con una leche tratada para expresar beta-galactosidasa, en las condiciones aquí planteadas, se asocia a un perfil de deposición grasa aumentado.

En definitiva, la alteración de la concentración de glúcidos en leche y en particular, la reducción de la concentración de lactosa y galactosa que se ha conseguido mediante la transfección in vivo en glándula mamaria de un enzima recombinante con actividad ß-galactosidasa recombinante, perturba el balance energético de las madres y de las crías y pone en evidencia mecanismos activos en la glándula mamaria para hacer frente a la inducción de esta expresión génica transitoria y de índole externa. Las adaptaciones en el organismo materno se asocian a una menor utilización de las reservas grasas para la síntesis de leche; las de la progenie, indican una influencia de la nutrición en fases tempranas de la lactancia que condiciona una programación metabólica hacia

fenotipo adulto de mayor eficiencia energética. En ambos casos se potencia el sobrepeso/obesidad.

Aunque estos resultados no son directamente extrapolables a humanos, ponen de manifiesto la relevancia de estudios a largo plazo para analizar la influencia de leches con contenido reducido en lactosa ingeridas desde edades tempranas. También apuntan a que este procedimiento, en las condiciones aplicadas, no sería el más adecuado para aplicar a animales de granja con la finalidad de producir leche con contenido reducido en lactosa.

II. INFLUENCIA DE LA SUPLEMENTACIÓN DE LA DIETA MATERNA CON LEUCINA

La leche es producida por la glándula mamaria cuyos genes son susceptibles de modulación y de adaptación (Rudolph et al., 2010), como también ha quedado demostrado en el estudio del impacto de la reducción de lactosa arriba comentado. Es indudable que la lactancia materna determina el correcto desarrollo de la descendencia y también condiciona la propensión a determinadas enfermedades, y por consiguiente, las modificaciones que se produzcan en la glándula mamaria influyen en la composición de la leche materna, y pueden afectar a la salud de la progenie, incluso en la edad adulta. (de Moura and Passos, 2005, Jensen and Lapillonne, 2009). Por otro lado, los hábitos dietarios de la madre y los suplementos en su alimentación durante la lactancia, afectan a la composición de la leche y también pueden incidir en el crecimiento y desarrollo de la descendencia (Chierici et al., 1999). Una mejor comprensión del metabolismo de la glándula mamaria y de su respuesta a factores externos, entre ellos la dieta, es un aspecto clave de la lactancia materna; solamente desde esta perspectiva es factible mejorar la composición de la leche y así contribuir al desarrollo óptimo del lactante.

La leucina es un aminoácido esencial presente en la leche, tanto formando parte de las proteínas lácteas (en su papel más tradicional y conocido) como también en forma libre y soluble en la leche, donde podría ejercer algún papel modulador *per se*. Así, recientemente se ha implicado a la leucina en la vía de señalización mTOR y, en el hipotálamo se ha propuesto que podría contribuir a modular el balance energético (Cota et al., 2006) Además, existen bastantes evidencias que describen efectos beneficiosos

de la suplementación con leucina, por ejemplo en la promoción de la síntesis de proteínas (Bolster, Vary et al. 2004, Matsuzaki, Kato et al. 2005, Layman y Walker 2006, Appuhamy, Knoebel et al. 2012), actividad intensa en los lactantes; y también en su contribución anti-obesidad en modelos animales, fomentando la pérdida de masa grasa y reduciendo la pérdida de tejido magro en dietas de adelgazamiento (Zhang, Guo et al. 2007, Chen, Simar et al. 2012, McAllan, Cotter et al. 2012). Por otra parte, datos previos de nuestro laboratorio han demostrado que la suplementación con leucina (2%) en ratas durante la lactancia mejora el perfil de su composición corporal por un mecanismo que implica la modulación de la expresión de genes orexigénicos a nivel hipotalámico (López, Sánchez et al. 2010).

Caracterización del perfil de expresión génica en glándula mamaria de ratas lactantes suplementadas con leucina

Dados los efectos descritos inicialmente sobre la composición corporal materna de la suplementación con leucina, nos planteamos caracterizar el impacto de dicha suplementación sobre el panorama metabólico de la glándula mamaria, analizando a nivel de expresión génica las principales vías metabólicas. Los resultados han mostrado un impacto significativo de la suplementación con leucina en el metabolismo de la glándula mamaria.

Es interesante destacar, que a pesar de promover una mayor disponibilidad de leucina dietaria, la expresión de su transportador LAT1, responsable de aminoácidos de cadena ramificada, leucina entre ellos, y de los aromáticos no se ha mostrado alterada; al igual que tampoco se han observado diferencias asociadas al tratamiento la expresión del transportador de aminoácidos neutros de cadena larga (ASC) ni la del transportador de aminoácidos catiónicos (CAT1). La expresión de estos transportadores varía de manera diferencial entre el embarazo y la lactancia y la captación de los aminoácidos correspondientes se ajusta a los requerimientos de la glándula mamaria que puede modular la expresión de dichos transportadores (Alemán, López et al. 2009). Así pues, la suplementación de leucina en la dieta materna en las condiciones estudiadas, no tiene una gran repercusión en la expresión génica de los transportadores de aminoácidos en glándula mamaria, pero si que repercute en una menor expresión del gen (CSN1S1) responsable de la síntesis de caseína, aunque la concentración de proteína total en

leche no se ve alterada por la suplementación. Estos resultados muestran una potencial influencia sobre la calidad de la leche, ya que esta familia de fosfoproteínas se almacena y secreta en forma de complejos de fosfato de calcio, formando y dando estabilidad a las micelas proteicas de la leche (Dalgleish y Corredig 2012)(Le Parc, Leonil et al. 2010).

La lactosa es el compuesto mayoritario en la leche, un disacárido formado a partir de UDP- galactosa y glucosa y constituye la principal fuente de energía en la leche (Jensen, 1995). Al analizar el gen determinante de la síntesis de lactosa (el complejo L-alba) se ha observado que los niveles de expresión del mismo son significativamente menores en la glándula mamaria de las madres tratadas. Este hecho puede provocar que la concentración de la lactosa en leche sea menor en estos animales, y que de forma paralela a lo que sucede en el planteamiento anterior las crías evolucionen hacia un mayor peso y/o masa grasa en la edad adulta. De hecho, el contenido de lactosa en leche también se ha visto reducido en estas madres bajo la suplementación dietaria. La disminución de la síntesis de lactosa además se ha asociado con una disminución en los niveles del transportador de glucosa GLUT1 en la glándula. Éste es principalmente sensible a la glucosa y la galactosa, lo que le hace directamente responsable de la utilización de sustratos por parte de la glándula en el proceso de síntesis de la lactosa. Estos resultados demuestran que el tratamiento dietario con leucina ha provocado una disminución en el trasiego de los azúcares, clave del metabolismo de la glándula mamaria hasta el punto de alterar la composición de la leche en su compuesto mayoritario. Es destacable que, a diferencia de lo que ocurre con el GLUT1, la expresión de GLUT4, transportador de glucosa sensible a la insulina, no se ve alterada. De acuerdo con ello, la expresión de los receptores de insulina en la glándula mamaria tampoco ha mostrado alteraciones, por lo que la glándula mamaria no desarrolla mecanismos compensatorios relacionados con la insulina en este aspecto. Este detalle es de relevancia, pues demuestra que de los dos puntos susceptibles en el metabolismo y utilización de la glucosa por la glándula, sólo en aquel independiente de insulina y que afecta de forma directa a las necesidades de sustratos en la síntesis de lactosa, la glándula manifiesta cambios, y concretamente reduce el potencial de movilización de dichos sustratos, lo que conduce a un menor contenido de lactosa en la leche de los animales suplementados con leucina.

La glándula mamaria es uno de los tres órganos principales en la síntesis de lípidos, junto al hígado y el tejido adiposo (Wakil 2009). Entre los genes analizados relacionados con el transporte y el metabolismo de los ácidos grasos, la suplementación con leucina muestra una disminución en la expresión de la Acly (ATP citrato liasa) y la SCD2 (esteraoil desaturasa). Ambos genes tienen un carácter lipogénico, lo que indica que la síntesis de novo de los ácidos grasos podría verse disminuida. En el ciclo de Krebs, ruta oxidativa e indispensable para el equilibrio metabólico, la enzima piruvato carboxilasa (PC), presenta del mismo modo niveles disminuidos de su expresión en la glándula mamaria. Los resultados manifiestan disminución en el metabolismo de síntesis, especialmente en aquellos puntos que conllevan un gasto energético. Otros genes relacionados con el transporte de ácidos grasos, como el FABP3 o el Slc27a3 han mostrado la misma tendencia aunque las diferencias respecto al control no son significativas. En conjunto, el metabolismo de la glándula, asociado a la suplementación con leucina promueve un ahorro de energía en las principales rutas, y la evidente disminución en la utilización de macronutrientes.

Influencia de la intervención nutricional temprana en el potencial de células "brite"

El crecimiento y desarrollo de las crías, y las modificaciones fenotípicas asociadas que se instauran ya desde edades tempranas, configuran lo que llamamos *programación metabólica* (Patel 2009), que explica el hecho de que la nutrición del lactante en edades tempranas esté asociada a la predisposición a padecer determinadas enfermedades metabólicas, entre ellas obesidad, en edad adulta (Li et al., 2008, Patel et al., 2009, Pico et al., 2011, McCrory and Layte, 2012). La intervención nutricional realizada sobre la madre mediante la suplementación con leucina durante la lactancia se ha asociado a cambios metabólicos en la progenie.

Recientemente ha resurgido el interés por el papel de los adipocitos marrones en el control de la obesidad. Como se sabe, el tejido adiposo está integrado por dos tipos de adipocitos, los blancos que regulan el balance energético mediante la secreción de leptina, y los marrones, que contribuyen al gasto de energía a través de la modulación de la UCP1. Aunque cada tipo de adipocitos se encuentra generalmente localizado en diferentes áreas, el descubrimiento de la presencia de adipocitos marrones infiltrados en

depósitos de tejido adiposo blanco, ha revelado que ante ciertos estímulos puede inducirse un proceso de "marronización" en el tejido adiposo blanco clásico. Estos adipocitos que presentan características específicas y solapadas entre los blancos y los marrones típicos, se han denominado "brite" (brown-in-white) y abren una interesante estrategia terapéutica en el tratamiento de la obesidad (Nedergaard et al., 2007, Sharp et al., 2012, Waldén et al., 2012), ya que estas células adipocíticas pueden contribuir a atenuar la tendencia de acumular depósitos de grasa ante una dieta obesogénica (Lasar 2013).

El tejido adiposo subcutáneo, y en especial el tejido adiposo inguinal, representa uno de los depósitos con mayor expresión de marcadores de células "brite" (Wu et al., 2012), de modo que ha sido el depósito de nuestra elección en este estudio de la descendencia.

En este contexto nos planteamos estudiar la influencia de la intervención nutricional temprana basada en la suplementación de leucina en la dieta de la madre, sobre el potencial "brite" en la progenie adulta y además determinar su capacidad ante un estímulo dietético obesogénico.

Los resultados revelan dos aspectos no descritos previamente: por una parte que la expresión de biomarcadores "brite" en el tejido adiposo inguinal se ve más infuenciada por la nutrición temprana (bajo suplementación materna con leucina) que por una dieta hipercalórica en edad adulta, y por otro lado que existe un patrón de expresión diferencial asociado al sexo, como ocurre con otros procesos metabólicos (Karastergiou et al., 2012). En el estudio de marcadores "brite" en función del sexo los machos muestran una notoria mayor expresión de los mismos.

La expresión de CIDEA, gen estructural de adipocitos marrones presentes en células brite se ha visto aumentada en los machos con respecto a las hembras. Además la ingesta de una dieta hiperlipídica también produjo un incremento en la expresión de este gen, y la suplementación materna con leucina dio lugar también al aumento de la expresión del CIDEA en las hembras. El Tbx15, un marcador de adipocitos "brite", fue detectado en las muestras analizadas, pero la elevada variabilidad en su expresión parece indicar que no es sensible a ninguna condiciones analizadas. La UCP1, un

marcador de tejido adiposo marrón, y el Prdm16, un coregulador transcripcional que controla el desarrollo de adipocitos marrones, no han sido detectados en este tejido. Sin embargo, dos de los genes caracterizados como marcadores de "brite", Hoxc9 y Shoxc2, manifestaron también una marcada diferencia en su expresión entre sexos, siendo considerablemente más elevada en los machos.

Se ha caracterizado también la expresión de otros genes del tejido adiposo, con el objeto de profundizar en las adaptaciones metabólicas y la influencia de las células "brite" en el depósito inguinal. La mayoría de los genes estudiados presentaron una influencia del sexo, con mayor expresión en los machos que en las hembras, como se observa en la expresión de biomarcadores de marrón o "brite". La expresión de algunos de estos genes podría estar asociado a la nutrición en edades tempranas, y sólo la expresión de Fasn se ha visto alterada, manifestando una disminución, en los animales alimentados con una dieta hiperlipídica. La leptina, que tiene un relevante papel en el balance energético y la ingesta, ya que aumenta en función de la tejido graso del individuo, muestra un incremento en su expresión en el depósito inguinal de los animales tratados con la suplementación materna de leucina, especialmente en las hembras. Los niveles de SERBP y PPARγ, factores de transcripción que modulan la expresión de otros genes (entre ellos el CIDEA) (Puri et al., 2008), reflejan el impacto de la nutrición temprana en la suplementación materna con leucina, ya que su expresión se vio aumentada en estos animales. Respecto a la UCP2, ésta también se vio afectada por la suplementación con leucina en la dieta materna, especialmente en las hembras, que presentaron menores niveles en su expresión. La expresión de CPTm, que promueve el transporte de ácidos grasos en la mitocondria e induce la producción de energía a partir de ellos, también mostró una reducción en los animales tratados.

En definitiva, los resultados aportados por la presente Tesis confirman el papel de la glándula mamaria, que como órgano encargado de la producción de leche determina la composición de la misma, y evidencian la capacidad plástica de la misma a sufrir adaptaciones ante estímulos nutricionales externos. Por otra parte la lactancia ejerce una programación temprana en las crías, y determina su metabolismo en edad adulta, de manera que se conserva una predisposición a padecer obesidad u otras enfermedades metabólicas. Además las diferencias observadas en el potencial "brite"

entre machos y hembras ponen de manifiesto la necesidad de tratar la obesidad de manera diferenciada entre sexos.

VI. Conclusiones

1. La obtención de leche materna reducida en lactosa puede llevarse a cabo mediante un método de transfección *in vivo* en la glándula mamaria de ratas gestantes, que permite expresar el gen de la β-galactosidasa en el tejido mamario de manera transitoria y produce la hidrólisis de la lactosa y consecuentemente una reducción en su contenido en leche.

2. La ingesta de esta leche con menor contenido en lactosa durante la lactancia condiciona una programación metabólica de la descendencia hacia un genotipo obeso, tal como se observa por el aumento de peso y una mayor cantidad de masa grasa en edad adulta, tanto en la progenie macho como en las hembras.

3. La suplementación con el aminoácido L-leucina en la dieta de madres lactantes, promueve una disminución en la expresión de genes clave del metabolismo en la glándula mamaria, especialmente en aquellos implicados en procesos de biosíntesis.

4. El tratamiento con suplementación de L-leucina en la dieta, causa en glándula mamaria una menor expresión en el gen responsable de la síntesis de la lactosa (α-lactalbúmina), el azúcar mayoritario de la leche que conlleva una disminución en el contenido de lactosa en leche.

5. En el análisis de marcadores "brite" en el tejido adiposo inguinal de ratas adultas, se ha puesto en evidencia el mayor potencial presente en machos respecto al de hembras, lo que condicionaría una capacidad de llevar a cabo un proceso de "marronización" sexo-dependiente.

6. Una intervención con dieta hiperlipídica durante 3 meses en ratas en edad adulta produce poco impacto en la expresión de marcadores "brite" en el tejido adiposo inguinal.

7. La suplementación con L-leucina en la dieta materna durante la lactancia, conlleva un aumento de los niveles de expresión de algunos marcadores “brite” y diferencias en la expresión de genes clave del metabolismo en el tejido adiposo inguinal de la descendencia en edad adulta; poniendo de manifiesto el impacto de la dieta materna sobre la programación perinatal de la progenie, particularmente en las hembras.

VII. Materiales y métodos

Obtención del material genético

Se utilizó el plásmido comercial *pTarget*^{TM} (5670bp) (Promega, Madison, WI) que contiene una versión modificada de la secuencia que codifica para el α-péptido de la β-galactosidasa, con una región promotora/activadora derivada del citomegalovirus humano que permite la expresión del DNA insertado en dicho plásmido en células de mamífero de forma constitutiva. Con dicho plásmido se transformaban bacterias E.Coli de la cepa JM109 (Promega, Madison, WI), se seleccionaban y se cultivaban para su crecimiento de acuerdo con las especificaciones proporcionadas por la casa comercial. Una vez seleccionado el clon correcto las bacterias se cultivaban en un medio adecuado compuesto por NaCl, extracto de levadura y triptona, en la proporción 5/5/10 respectivamente, al que se le añadía ampicilina, en una concentración final de 0,1 mg/ml. A las 24 h se recogían por centrifugación a 6000g 15 min. y a 4ºC. De dichos cultivos se aislaba y se purificaba el plásmido siguiendo el protocolo de purificación estándar *Plasmid Maxi Kit* (Quiagen, Madrid, Spain), basado en su liberación al medio mediante lisis alcalina de las bacterias que lo contienen y su atrapamiento en una columna de intercambio aniónico que facilita la eliminación de impurezas: El sedimento bacteriano obtenido tras la centrifugación se resuspendía en un tampón Tris que contenía ARNasa A. Acto seguido se añadía una solución de NaOH y SDS que provocaba la lisis bacteriana. Después se añadía un tampón con acetato potásico que precipita material celular diverso, y deja un sobrenadante con el ADN plasmídico. Tras dos centrifugaciones a 20000g se recogía el sobrenadante que contenía el plásmido. La purificación del mismo se llevaba a cabo mediante una columna de intercambio aniónico; se procedía a los sucesivos lavados de la columna y en el paso final se recogía el DNA plasmídico con una solución de NaCl, Tris base e isopropanol (pH 8,5). El DNA plasmídico entonces se precipitaba con isopropanol, que permite su concentración y disolución en las condiciones adecuadas para su uso en la posterior transfección. Después se procedía a un lavado con etanol al 70%. Finalmente, el plásmido se resuspendía en agua libre de ARNasas (Sigma) y se determinaba su concentración por espectrofotometría con un *NanoDrop® Spectrophotometer ND-1000* (NanoDrop Technologies, Wilmington, DE, USA). La secuenciación del plásmido se realizaba de manera automática por la empresa Secugen SL.

Inyección del plásmido

Se utilizaron ratas hembras vírgenes de la raza *Wistar* (Charles River Lab, Barcelona), de 200 gramos de peso aproximadamente, estabuladas con un ciclo de luz/oscuridad de 12 horas (luz de 8:00 a 20:00), con temperatura regulada a 22º y alimentadas *ad libitum* con pienso estándar. Las ratas fueron apareadas con machos controles y para determinar el día de la concepción se valoró la presencia de esperma mediante examen del flujo vaginal. A partir de este momento y durante la gestación, las ratas fueron mantenidas en jaulas individuales con libre acceso al agua y al pienso. Entre 2 y 4 días antes del parto previsto, se llevó a cabo una inyección subcutánea en el tejido mamario de 50 μl de la solución con el plásmido, en cada una de las 12 glándulas mamarias y en condiciones de anestesia con éter. El plásmido inyectado había sido diluido en PBS para conseguir una concentración de 1μg/μl y acomplejado con una solución de DEAE-dextrano en un ratio de 1,39 DNA/DEAE-dextrano (Sigma, Sant Louis, Missouri). La mezcla para la transfección fue atemperada a 37º antes de la inyección.

Medidas antropométricas

El peso y la ingesta se midieron periódicamente. La composición corporal se realizó mediante el analizador de composición corporal específico Echo MRI en los días indicados.

Recogida de sangre y procesamiento de la sangre

La sangre se recogía directamente de la pata mediante una pequeña incisión en la vena con material heparinizado (0.2% heparina diluida con suero salino, Sigma) o no (según conveniencia). Para la obtención del plasma o suero la sangre se centrifugaba a 1000g durante 10 min. y a 4ºC. Posteriormente el plasma se guardaba a -20º hasta su utilización.

Recogida de la leche

Las crías se separaban de las madres entre 3-4 horas antes de la extracción para permitir el cúmulo de mayor cantidad de leche. En condiciones de anestesia con éter, la leche se recogía por ordeñe de cada una de las mamas y se juntaban para su conservación en el mismo tubo. Posteriormente se conservaba a -70º hasta su análisis.

Determinación de la concentración de leptina

Los niveles de leptina se medían en leche mediante el inmunoensayo enzimático (ELISA) con el kit *Quantikine Mouse Leptin Inmmunoassay* (DRG Diagnostics) siguiendo las instrucciones del fabricante. Se introducían 50 μl de las muestras (previamente diluidas según el caso entre 1/2 a 1/5) y los diferentes puntos de patrón en cada uno de los pocillos de las placas suministradas por el kit, que están recubiertas con un anticuerpo policlonal anti-leptina, y se incubaban durante 2h a temperatura ambiente. A continuación se hacían 5 lavados con el tampón suministrado por el kit, se añadía conjugado anti-leptina en cada pocillo y se incubaba nuevamente 2h a temperatura ambiente. Posteriormente, se hacían 5 lavados más, se añadía la solución sustrato y se incubaba entonces 30 minutos a temperatura ambiente y oscuridad. Finalmente se añadía la "solución de parada" de la reacción y se leía la absorbancia en los pocillos a 450 nm, utilizando un espectrofotómetro de placas de ELISA *Sunrise* de TECAN.

Reactivos:

- Kit Quantikine Mouse *Leptin Immunoassay* (DRG Diagnostics).
- PBS, pH 7,4: NaCl (Panreac) 137 mM, KCl (Panreac) 2,7mM y fosfato sódico mono-/dibásico (Panreac) 10mM.

Determinación de glucosa

Los niveles de glucosa en leche se determinaron usando un kit comercial (Roche-diagnosis) basado en un método enzimático. Las muestras de leche eran previamente desproteinizadas mezclando en una proporción 1:10 de leche con ácido perclórico 0,33M. Las muestras se mezclaban vigorosamente y se centrifugaban en frío durante 20 min. a 3000 rpm. Seguidamente, se recogía el sobrenadante y se neutralizaba añadiendo KOH-CO_3HK 2N (se añadían 0,5 ml de KOH-CO_3HK 2N por cada ml de ácido perclórico usado en la desproteinización) y se centrifugaba 10 min. a 3000rpm y a 4ºC. Del sobrenadante se llevó a cabo la determinación.

Para determinar la concentración de glucosa se seguían las instrucciones del kit: a 50 μl de las muestras o del correspondiente punto de patrón se le añadían 500 μl de H_2O, 250 μl de la solución 1, y 25 μl de la solución 2. Se mezclaba bien y se dejaban reposar las muestras durante 30 min. a temperatura ambiente y en condiciones de oscuridad. Pasados los 30 min.

las muestras se agitaban por inversión y se medían las absorbancias a 340 nm, utilizando un espectrofotómetro de placas de ELISA *Sunrise* de TECAN, tanto de las muestras como del patrón que se suministraba el kit.

Reactivos:

- Ácido perclórico (Panreac) 0,3M
- Kit para la determinación de D-Glucosa (Roche)
- KOH-CO_3HK (Panreac) 2N

Los niveles de glucosa en sangre se median mediante un *Accu Check Sensor* (Roche Diagnostics, Barcelona).

Determinación lactosa y galactosa

Los niveles de lactosa y galactosa en leche se determinaron usando un kit comercial colorimétrico basado en la medida de la galactosa producida tras la hidrólisis enzimática de la lactosa.

Para la determinación se seguían las especificaciones del kit: Se añadían 990 μl de *Lactose Assay Buffer* a 10 μl de la solución *Lactose Standard* (100nmol/μl). A esta solución se le añadían 2, 5, 10, 15, 20, 25 y 50 μl para establecer los puntos de patrón. Se mezclaba bien y se ajustaban todos los volúmenes a 50 μl por pocillo con el *Lactose Assay Buffer.* Se diluían las muestras de leche a 1/1000 con el mismo *Lactose Assay Buffer,* y se llevaban también a un volumen final de 50 μl. Se añadían 2 μl de lactasa en cada pocillo, que convierte la lactosa en glucosa y galactosa. Se añadían 50 μl de *Reaction Mix* previamente preparada con: 44 μl de *Lactose Assay Buffer*, 2 μl de *Probe* (solución de DMSO como solvente), 2 μl de *Lactose Enzyme Mix* y 2 μl de HRP (peroxidasa que cataliza la reacción). Tras mezclarlo bien se incubaba durante 60 minutos a 37º y protegido de la luz. Pasado ese tiempo se medía por absorbancia a 570nm utilizando un espectrofotómetro de placas de ELISA *Sunrise* de TECAN. La cantidad de lactosa se calculaba como la cantidad de galactosa final (producto de la hidrólisis) menos la galactosa inicial.

Reactivos:

- Kit lactosa (*Lactose Assay Kit*) (Biovision, Deltaclon)

Test de tolerancia a la glucosa

Se administraron 1,5 g de glucosa por kg de peso del animal, en condiciones de ayuno, y al comienzo del ciclo de luz. Se recogía la sangre según lo mencionado anteriormente, y se medían las concentraciones de glucosa en sangre a tiempo 0, 30, 60, 120 y 180 minutos tras la carga de glucosa mediante la utilización del glucómetro con tiras reactivas *Accu Check Sensor* (Roche Diagnostics, Barcelona).

Extracción y cuantificación del ARN

Para la extracción del ARN se utilizaba un reactivo comercial basado en fenol e isotiocianato de guanidina que permite la lisis de las estructuras celulares manteniendo la integridad del ARN (Tripure, Roche). Se siguieron las instrucciones de la casa comercial: Las muestras de tejido se homogeneizaban con Tripure (1 ml de Tripure por cada 100mg de tejido) y un homogenizador por dispersión VDI12. Como paso previo al protocolo de extracción del ARN, las muestras adiposas se centrifugaban a 12000g durante 10 min. para eliminar la grasa. Para la separación del ARN se añadían 200 μl de cloroformo (Sigma) a las muestras y se agitaba vigorosamente durante 15 s. Las muestras se dejaban reposar unos 5-15 min. en frío y posteriormente se centrifugaban 15 min. a 12000g y 4ºC, recogiendo a continuación la fase acuosa superior, transparente, que contenía el ARN mientras que la fase inferior, en la que se encontraban las proteínas y el ADN, se guardaba a -20ºC. Para la precipitación del ARN se añadían 500 μl de isopropanol (Sigma) y se agitaba ligeramente por inversión. Las muestras se dejaban reposar al menos 10 min. a temperatura ambiente o a -20ºC toda la noche. Después, las muestras se centrifugaban a 12000g durante 15 min. y se obtenía así un precipitado de ARN. Se retiraba el isopropanol, se añadía 1 ml de etanol al 75% (Panreac) y se agitaba vigorosamente durante 15 s. A continuación se realizaba una centrifugación, esta vez a 7600g durante 5 min., obteniéndose así el precipitado final de ARN. Una vez eliminado el etanol, el ARN se resuspendía en un volumen apropiado (entre 20 y 100 μl) de agua libre de ARNasas (Sigma). Para facilitar la resuspensión del ARN, las muestras se podían calentar en un baño termostatizado a 60ºC. El ARN extraído se guardaba para su correcta conservación a -70ºC.

Cuantificación y valoración del estado del ARN

Cuantificación del ARN. El ARN se cuantificaba por espectrofotometría (*NanoDrop* ND-1000), valorando la absorbancia de las muestras a 260nm. Una unidad de absorbancia a 260 nm corresponde a 40 μg de ARN por ml. Se realizaba también una medida a 280 y 230 indicativos del grado de contaminación por proteínas y por disolventes orgánicos respectivamente. Un ARN puro tiene valores de entre 1,8-2,0 para el ratio 260/280 y de 1,8-2,2 para el ratio 260/230.

Valoración de la integridad del ARN. La integridad del ARN se comprobaba mediante la carga de 0,5 μg de ARN, previamente mezclados con un tampón de carga, en un gel de agarosa al 1% (Pronadisa) con bromuro de etidio o *SYBER Safe* (Invitrogen). La visualización de dos bandas correspondientes a los ARNs ribosomales 28S y 18S son indicativos de un ARN en buen estado mientras que un difuminado de bandas indica que el ARN está degradado.

Tampones:

- Tampón de electroforesis: TBE 0.5 x.
- Tampón de carga: glicerol, azul de bromofenol

RT-PCR

Retrotranscripción (RT)

Para la detección de los ARN de interés se usaban 0.25 μg de ARN en el caso de la glándula mamaria y 0.08 μg en el caso del tejido adiposo que se llevaban a un volumen total de 5 μl con agua libre de ARNasas (Sigma) y se desnaturalizaba 1 min. a 90º. Para llevar a cabo la RT se añadían 10 μl de RT-mix, preparada para que las concentraciones finales en cada tubo de reacción fueran: 1μl de 10x buffer (Promega), 2,5 mM de MgCl2 (Promega), 0,4 mM de nucleótidos (Invitrogen), 5 μM de *random hexamers* (Applied Biosystems), 5 μM de inhibidor de ARNasas (Applied Biosystems) y 2,5 U/μl de transcriptasa inversa (MuLV RT, *murine leukemia virus reverse transcriptase*, Applied Biosystems). Las condiciones de retrotranscripción eran: 60 min. a 42ºC y un paso final de 5 min. a 99ºC. Todas las reacciones se llevaron a cabo en un termociclador *Perkin Elmer 9700.*

Reacción en cadena de la polimerasa PCR a tiempo real

Se utilizaban 2 µl del producto de RT diluido (entre 1/5 a 1/50, dependiendo de la abundancia del gen problema en la muestra). A la muestra se le añadían 9 µl de una mezcla de PCR-mix que contenía: 3,1 µl de agua libre de ARNasas, 0,45 de cada cebador (a una concentración que dependía de la abundancia de cada ARNm en un determinado tejido, entre 10-20 µM) y 5 µl de una mezcla comercial *Power SYBR Green PCR Master Mix* (Applied Biosystems). Para realizar la PCR se utilizaba el termociclador *StepOnePlus™* (Applied Biosystems) con el siguiente perfil: 10 min. a 95ºC, seguido de un total de 40 ciclos donde había una desnaturalización durante 15 segundos a 95ºC seguida de una elongación de 1 min. a 60ºC. Con el objetivo de verificar la pureza de los productos obtenidos, se realizaba una curva de desnaturalización después de cada PCR realizada, siguiendo las instrucciones de la casa comercial. Además, una vez finalizada la reacción se verificaba el tamaño del fragmento amplificado en un gel de agarosa al 2% teñido con bromuro de etidio o *SYBER Safe* (Invitrogen) que se visualizaba con luz ultravioleta.

RT-PCR para determinación de la expresión de los miRNAs

El ARN extraído con Tripure, conforme se explica en el apartado de aislamiento, se llevaba a una concentración de 2,5 ng/µl. Para cada miRNA se necesitaba realizar una RT en la que se utilizaba 2 µl de la dilución de ARN a la que se añadía 4 µl de la mix (*TaqMan® MicroRNA Reverse Transcription kit*, Applied Biosystems). Para la transcripción de utilizaba un termociclador *Perkin-Elmer 9700* (PerkinElmer, Wellesley, MA) con las siguientes condiciones: 30 min. a 16ºC, seguido de 30 min. a 42ºC y finalmente 5 min. a 85ºC. Finalizada la RT, 2 µl del DNA complementario específico de cada miRNA se utilizaba para la reacción de PCR utilizando en este caso sondas *TaqMan* (Applied Biosystems) conjuntamente con la mezcla comercial *TaqMan Universal PCR master mix* (Applied Biosystems). La reacción de PCR se realizó utilizando el termociclador *StepOnePlus™* (Applied Biosystems) en las siguientes condiciones: 95ºC durante 10 min., seguido de 40 ciclos (desnaturalización a 95ºC durante 15 s, hibridación y extensión 60ºC durante 1 min.).

ANÁLISIS ESTADÍSTICO

Para el análisis estadístico se utilizaba el programa SPSS para Windows. En función del tipo de variable y los grupos de estudio las diferencias se determinaban utilizando el análisis de la media por *t-Student* o por análisis de la varianza (ANOVA) uni o multivariante según el caso. En la mayoría de los casos se establecía una homogeneidad de varianzas mediante la prueba de Levene. En todos los casos el nivel de confianza considerado era 95% ($P<0,05$) o superior.

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