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EFECTOS DE LA PROLACTINA PROVENIENTE DE LA LECHE MATERNA EN LA PREVENCIÓN DE OBESIDAD Y ALTERACIONES METABÓLICAS EN LAS CRÍAS

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LISTA DE ABREVIATURAS

5-HT	5-hidroxitriptamina o serotonina
ADN	Ácido desoxirribonucleico
AGNEs	Ácidos grasos no esterificados
ARH	Núcleo arqueado del hipotálamo
ARN	Ácido ribonucleico
AUC	Área bajo la curva
CD	Dieta control
DHA	Ácido docosahexaenoico
EDTA	Ácido etilendiaminotetraacético
EGF	Factor de crecimiento epidérmico
EGTA	Ácido egtazico
ENSANUT	Encuesta Nacional de Salud y Nutrición
EPO	Eritropoyetina
$FADH_2$	Flavín adenín dinucleotido reducido
FGF21	Factor de crecimiento de fibroblastos
FITC	Isotiocianato de fluoresceína
GCG	Glucagon
GH	Hormona de crecimiento
GLP-1	Péptido parecido al glucagon 1
GLUT-4	Transportador de glucosa 4
GM	Glándula mamaria
H&E	Hematoxilina y eosina
HFD	Dieta alta en grasas
HTR7	Receptor de 5-hidroxitriptamina
IGF	Factor de crecimiento insulínico
IKK	Cinasa de IKβ
IL-18	Interleucina 18
IL-1β	Interleucina 1 beta
IL-6	Interleucina 6
IMC	Índice de masa corporal
IR	Receptor de insulina
IRS-1	Sustrato del receptor de insulina
ITT	Ensayo de tolerancia a la insulina
JNK	Cinasa c-Jun N-terminal
LPS	Lipopolisacáridos
MAPK	Proteína cinasa activada por mitógeno
NADH	Dinucleótido de nicotinamida y adenina reducido
NF-κB	Factor nuclear potenciador de las cadenas ligeras kappa de las

	células B activadas
OMS	Organización Mundial de la Salud
PBS	Buffer de fosfatos
PKC	Proteína cinasa C
PL	Lactógeno placentario
PPARγ	Receptor gamma activado por el proliferador de peroxisomas
PRL	Prolactina
qPCR	Reacción en cadena de la polimerasa en tiempo real
ROS	Especies reactivas de oxígeno
RPRL	Receptor de Prolactina
Sc	Tejido adiposo subcutáneo
SDS/PAGE	Electroforesis en gel de poliacrilamida con dodecilsulfato sódico
Ser	Residuos de serina
SOCS-2	Supresor de la señalización de citocinas 2
STAT5	Transductor de señal y regulador de la transcripción 5
TAG	Triglicéridos
TGFβ1	Factor de crecimiento transformante beta 1
Thr	Residuos de treonina
THR	Hormona liberadora de tirotropina
TNF-α	Factor de necrosis tumoral alfa
TPH-1	Triptófano hidroxilasa
UPR	Respuesta a proteínas desplegadas
VEGF	Factor de crecimiento endotelial vascular
Vis	Tejido adiposo visceral

RESUMEN

La nutrición materna durante la lactancia ejerce una fuerte influencia sobre el metabolismo de su progenie y es un factor determinante para la salud a lo largo de su vida. Esta relación se establece a través de la leche materna, la cual contiene una gran variedad de componentes con propiedades funcionales. Tal es el caso de la hormona prolactina (PRL), que se encuentra presente en altas cantidades en la leche materna. En la vida adulta, la función principal de la PRL es la regulación y el mantenimiento de la lactancia, pero también ejerce efectos metabólicos como el incremento de la sensibilidad a la insulina en condiciones de obesidad. Sin embargo, la función de la PRL de la leche sobre el metabolismo de las crías lactantes se desconoce. Por lo que en este trabajo se evaluó si el consumo materno de una dieta alta en grasas (HFD) durante la lactancia, altera los niveles de PRL, tanto en el suero de la madre como en la leche, y si esta disminución contribuye con el daño metabólico de las crías inducido por la dieta materna obesogénica. Interesantemente, se encontró que la dieta alta en grasas resultó en una disminución de los niveles de PRL en la leche, pero no en el suero. Así mismo, el consumo materno de una HFD redujo los niveles de expresión del receptor de PRL (rPRL) en la glándula mamaria (GM) y alteró la estructura y función de la misma. En relación con las crías lactantes provenientes de las madres alimentadas con la HFD, estas resultaron con mayor ganancia de peso corporal y adiposidad, mayor acumulación de triglicéridos en hígado, hiperinsulinemia y resistencia a la insulina al destete. Mientras que al incrementar los niveles de PRL en las madres alimentadas con HFD (HFD+PRL), se normalizó tanto la función y la estructura de la GM, así como los niveles de PRL en la leche. Además, las crías de las madres HFD+PRL, así como crías de madres en HFD y tratadas a su vez con PRL oral durante la lactancia, mostraron una menor adiposidad visceral, menor acumulación de triglicéridos (TAG) en el hígado y mejoraron su sensibilidad a la insulina, más aún, presentaron mayor activación de la vía de señalización de PRL, vista como un incremento en la fosforilación de la proteína STAT5 en el hígado. En cuanto a los mecanismos propuestos, el consumo materno de una HFD incrementó la permeabilidad intestinal de las crías, mientras que el tratamiento con PRL en las madres o directamente en las crías, resultó en una disminución de la permeabilidad, lo cual puede estar asociado con la mejor sensibilidad a la insulina que presentaron las crías. Estos resultados demuestran que la HFD altera la función de la PRL durante la lactancia, lo cual se ve reflejado en efectos adversos sobre la fisiología de la GM, y en niveles disminuidos de PRL en la leche materna lo que a su vez genera daños metabólicos en las crías lactantes.

ABSTRACT

Maternal diet during lactation influences offspring metabolic health throughout life. This relationship is stablished by maternal milk, which contains a diversity of components with functional properties. In this regard, the hormone prolactin (PRL) is present in high quantities in maternal milk. PRL main function in adults is the regulation and maintenance of lactation, and also exerts multiple roles including metabolic actions, specifically improving insulin resistance in obesity conditions. However, the effects of milk PRL on the offspring metabolism remain poorly characterized. In this study, we evaluated whether feeding a high-fat diet (HFD) to rats during lactation alters PRL, both in the mother's serum and in milk, and whether this factor contributes to HFD-induced metabolic dysfunction in the offspring. Maternal HFD resulted in decreased PRL levels in milk, reduced mammary gland (GM) PRL receptor expression, and altered GM structure and function. Offspring from HFD-fed dams had increased body weight and adiposity, and increased triglycerides accumulation in liver, hyperinsulinemia, and insulin resistance at weaning. Increasing PRL levels in the HFD-fed mothers by subcutaneous osmotic minipumps releasing PRL normalized GM function and PRL levels in milk. Moreover, PRL treatment in HFD-fed mothers, or directly in their pups via oral PRL administration, increased PRL signaling, seen by liver STAT5 phosphorylation, reduced visceral adiposity, ameliorated triglycerides accumulation in liver, and improved insulin sensitivity in weaned offspring. Mechanistically, we found that maternal HFD intake, resulted in increased intestinal permeability in their offspring, whereas PRL treatment normalized permeability, which may explain the improved insulin sensitivity in the pups. Our results show that HFD impairs PRL actions during lactation reflected in impairment of GM physiology and that milk PRL directly affect the offspring metabolism.

1. INTRODUCCIÓN

La nutrición materna durante el embarazo y la lactancia es un factor determinante para la salud metabólica de su progenie. En los últimos años se ha demostrado que la exposición a un ambiente obesogénico durante etapas tempranas del desarrollo, promueve cambios metabólicos en las crías que pueden incrementar el riesgo de desarrollar obesidad y otras alteraciones metabólicas a lo largo de la vida.

Durante la lactancia la madre proporciona a través de la leche materna, los nutrientes necesarios para el desarrollo del infante, por lo cual es considerada como el alimento ideal durante los primeros años de vida. Sin embargo, se ha demostrado que la calidad de la leche materna puede ser modificada por diversos factores, lo que a su vez tendrá impacto sobre la salud metabólica de la progenie.

Dentro de los factores más importantes que determinan la composición de la leche materna, se encuentra la nutrición de la madre. Al respecto, se ha demostrado que el consumo de dietas altas en grasas y/o azúcares promueven el desarrollo de sobrepeso y obesidad en los primeros años de vida, además de promover otras alteraciones metabólicas que pueden derivar en el desarrollo de enfermedades en la vida adulta, como síndrome metabólico, diabetes, enfermedades cardiovasculares, entre otras.

La leche materna tiene una gran variedad de componentes que son considerados funcionales, debido a que proporcionan beneficios a la salud. Entre ellos se pueden mencionar hormonas, como la insulina, adiponectina, leptina, factor de crecimiento de fibroblastos 21 (FGF21), prolactina (PRL), entre otras. La mayoría de estas hormonas han sido estudiadas en relación con sus efectos metabólicos tanto durante la lactancia como en otras etapas de la vida. Sin embargo, la prolactina que es una de las hormonas más importantes para la regulación de la lactancia, no se ha investigado con relación a su función metabólica en las crías durante este periodo.

Se conoce que la PRL se encuentra en altas concentraciones en la leche materna, y que esta hormona presente en la leche puede llegar hasta la circulación de las crías lactantes. En cuanto a sus efectos metabólicos, se sabe que, en niños con síndrome metabólico, los niveles de PRL del suero se encuentran reducidos; mientras que al disminuir su peso corporal y mejorar su sensibilidad a la insulina mediante dieta y ejercicio, se encontró que los niveles de la PRL se incrementaron. En concordancia, se encontraron niveles reducidos de PRL en el suero de ratas adultas con obesidad inducida por dieta, las cuales al ser tratadas

con PRL, mostraron una mejor sensibilidad a la insulina. Estos hallazgos muestran una asociación entre la PRL y la homeostasis metabólica, sin embargo, se desconoce si durante la lactancia, la PRL de la madre, presente en la leche materna puede ejercer un efecto metabólico en las crías lactantes.

Por lo anterior en este trabajo de investigación se propuso evaluar los efectos de la PRL presente en la leche materna en la prevención de obesidad y alteraciones metabólicas en las crías provenientes de madres alimentadas con una dieta alta en grasas durante la lactancia.

2. ANTECEDENTES

2.1 Efectos funcionales de la lactancia materna

2.1.1 La práctica de la lactancia materna y sus efectos sobre la salud del lactante

La lactancia materna es definida por la Organización Mundial de la Salud (OMS) como la alimentación proporcionada por el seno materno y que proporciona los nutrientes necesarios para un crecimiento y desarrollo adecuados. Por lo cual la propia OMS recomienda la práctica de la lactancia materna exclusiva, durante los primeros 6 meses de vida y su continuación hasta los dos años o más en combinación con otros alimentos (Gonzalez de Cosio et al. 2013).

La práctica de la lactancia a nivel mundial ha disminuido drásticamente en los últimos años (Fig. 1) (Victora et al. 2016). En México la Encuesta Nacional de Salud y Nutrición 2012 (ENSANUT) reporta que tan solo un tercio de los recién nacidos son alimentados con leche materna en la primera hora de vida. Mientras que el porcentaje de niños menores de 6 meses de edad que reciben lactancia materna exclusiva es muy bajo. Otro dato alarmante es que entre los años 2006 y 2012 la práctica de la lactancia materna exclusiva disminuyó significativamente tanto en las zonas rurales, en donde pasó de un 36.9 a un 18.5%, y a nivel nacional bajo de un 22.3 a un 14.4% (Gonzalez de Cosio et al. 2013).

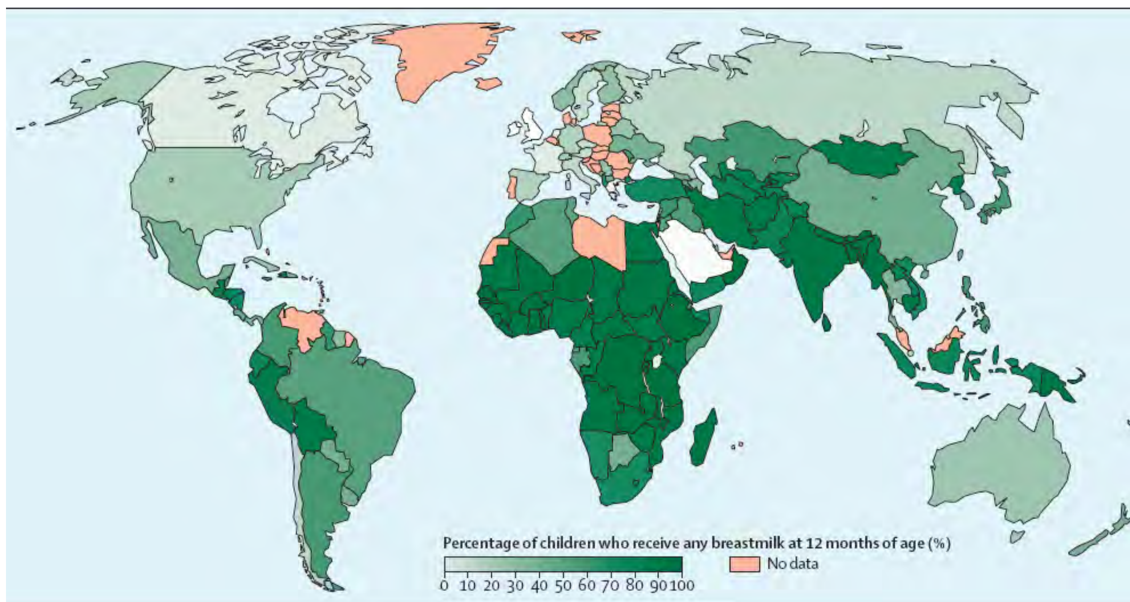

Fig. 1 Distribución global de la práctica de la lactancia materna en el primer año de vida. Datos de 1995 al 2013 (Victora et al. 2016).

La lactancia materna es considerada como una de las principales vías para prevenir el desarrollo de enfermedades en la población infantil. Se ha reportado que la leche materna previene la mortalidad infantil por enfermedades infecciosas como la diarrea, enfermedades respiratorias, enterocolitis necrotizante y también por el síndrome de muerte súbita infantil (Horta BL 2013, Sankar et al. 2015). Por otro lado, también se ha observado que los niños que fueron alimentados con leche materna poseen mayor coeficiente intelectual (Horta et al. 2015).

Así mismo, en los últimos años se ha incrementado la evidencia que apoya la relación entre la ausencia de lactancia materna y la obesidad. Algunos estudios han reportado que existe menor prevalencia de sobrepeso y obesidad en los niños que son alimentados con leche materna (Giugliani et al. 2015, Horta et al. 2015). Mientras que estudios en población infantil mayor (6-14 años) han mostrado menor prevalencia de sobrepeso y obesidad en aquellos alimentados con leche materna por periodos iguales o mayores a los 6 meses de edad (Toschke et al. 2002, Toschke et al. 2007).

La lactancia materna es el primer sistema de comunicación externo entre la madre y el infante, de ahí la gran relevancia que tiene este periodo con la información que la madre puede transferir a su hijo y viceversa. Por lo tanto las características maternas, tales como la salud, el estatus nutricional, el tipo de alimentación, los hábitos, entre otros, tienen influencia sobre el proceso de lactancia, incluida la composición de la leche; que a su vez repercute sobre la salud física y emocional del lactante (Nommsen et al. 1991, Ballard et al. 2013, Victora et al. 2016).

2.2 Obesidad y nutrición durante la lactancia

2.2.1 Prevalencia de sobrepeso y obesidad en mujeres en edad reproductiva

La prevalencia de sobrepeso y obesidad en mujeres se ha incrementado drásticamente a nivel mundial. De acuerdo con datos de la OMS, tanto en Europa como en América, más del 50% de las mujeres tienen sobrepeso y obesidad (Fig. 2). De manera alarmante, en México y Estados Unidos la prevalencia de sobrepeso y obesidad en mujeres es del 76%. Específicamente, en México, de la población de mujeres adultas un 37% tiene sobrepeso y un 38.6% padece obesidad. Así mismo de acuerdo a los datos de la Encuesta Nacional de Salud y Nutrición 2016 (Hernández-Avila M 2016) en los últimos 28 años, la prevalencia de sobrepeso en mujeres de entre 20 y 49 años, es decir en edad reproductiva, incrementó un 42.4%, mientras que la prevalencia de obesidad aumentó en un 290%. La tendencia muestra que el índice de mujeres en edad reproductiva con obesidad sigue en aumento, ya que se observó que la prevalencia de obesidad

aumentó en un 5.4% entre el año 2012 y 2016 (Fig. 3). Estas cifras son relevantes debido al impacto que tienen sobre la salud metabólica de su progenie, ya que existe una relación directa entre madre e hijo, establecida por la denominada programación metabólica (Perez Ferrer et al. 2014, Hernández-Avila M 2016).

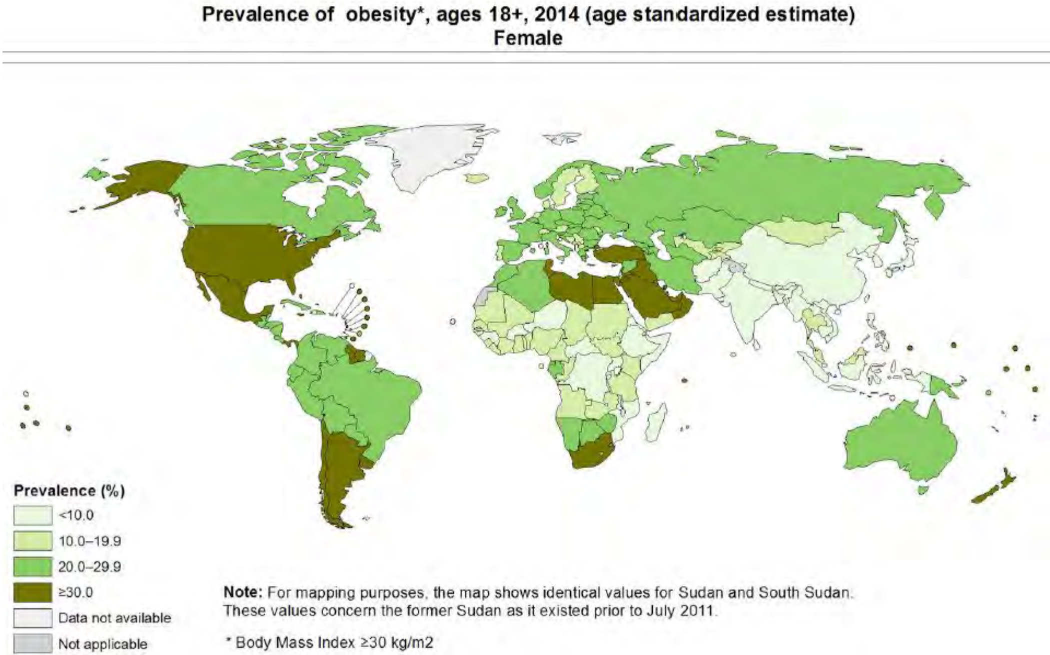

Fig. 2 Prevalencia mundial de obesidad en mujeres mayores de 18 años (WHO 2017).

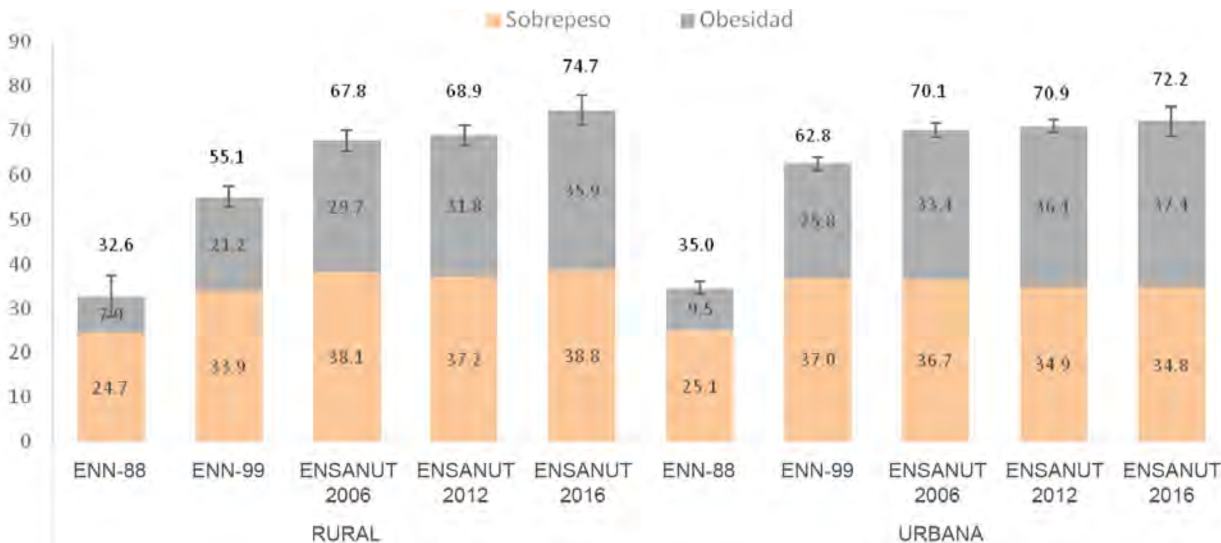

Fig. 3 Prevalencia histórica de sobrepeso y obesidad en las mujeres de 20 a 49 a. os de edad, categorizando por tipo de localidad en México. ENN-1988, ENN-1999, ENSANUT 2006, ENSANUT 2012 y ENSANUT 2016 (Hernández-Avila M 2016).

Durante los primeros años de vida, algunos factores de la madre y del entorno tienen influencia a corto y largo plazo sobre el fenotipo del infante, por ejemplo, el ambiente nutricional de la madre durante el embarazo y la lactancia, así como el tipo de alimentación en los primeros años de vida.

Específicamente, se conoce que la presencia de un ambiente obesogénico proveniente de la madre y/o de los primeros alimentos proporcionados al infante, en conjunto con la predisposición genética, promueven el desarrollo de obesidad y otras complicaciones durante la infancia y la vida adulta.

2.2.2 Nutrición materna y programación metabólica

Determinados periodos del desarrollo son críticos para el establecimiento de la salud, como el prenatal, la lactancia, la infancia y la pubertad, inclusive se ha reconocido como ventana crítica para el desarrollo el periodo preconcepcional. La relevancia de estas etapas se debe a que cualquier estímulo o insulto metabólico que ocurra, puede tener influencia a largo plazo, en otras palabras, ocurre una programación metabólica que marcará la salud metabólica futura (Patel et al. 2010, Desai et al. 2014).

Estudios en humanos han encontrado que la ganancia de peso en el embarazo influye positivamente con el índice de masa corporal de sus hijos a lo largo de su infancia y la vida adulta (Schack-Nielsen et al. 2010). Así mismo se ha encontrado que la obesidad materna promueve la acumulación de grasa en su progenie (Hull et al. 2011), y en conjunto con la sobrenutrición materna se asocian con mayor prevalencia de desarrollar obesidad en la infancia y la vida adulta (Ross et al. 2013).

Específicamente se ha observado que la obesidad materna y el consumo de dietas altas en grasa promueven mayor adipogénesis y la activación de vías hipotalámicas neurales para promover el apetito (Chen et al. 2009, Li et al. 2011).

Durante la lactancia, la programación metabólica se establece a través de la leche materna, por lo que su composición es determinante. Los componentes de la leche están fuertemente influenciados por el estatus metabólico materno, que a su vez deriva de la dieta, el peso corporal, el porcentaje de grasa, etc. Al respecto, se conoce que el consumo de dietas altas en grasa durante la lactancia altera la composición de la leche, lo cual tiene influencia sobre la programación metabólica del lactante (Rolls et al. 1986, Dewey 2003).

2.2.3 Efectos del consumo materno de una HFD durante la lactancia sobre el metabolismo de su progenie

El consumo de dietas altas en grasa (HFD) en ratas durante la lactancia, independientemente del tipo de dieta durante la gestación, produce alteraciones metabólicas en sus crías, representadas por mayor ganancia de peso durante la lactancia, mayor adiposidad, intolerancia a la glucosa, hiperleptinemia, entre otras, las cuales en conjunto con otras alteraciones como incremento en los niveles de triglicéridos en suero, reducción de los niveles de adiponectina circulante, y resistencia a la insulina continúan y se exacerban en la etapa adulta (Sun et al. 2012, Masuyama et al. 2014, Vogt et al. 2014, Du et al. 2015). Algunos trabajos han considerado que la nutrición materna durante la lactancia ejerce mayor influencia sobre el fenotipo metabólico de las crías que la alimentación de la madre durante la gestación (Sun et al. 2012, Masuyama et al. 2014).

Dentro de las complicaciones derivadas del consumo materno de dietas altas en grasa durante la lactancia presentadas en animales adultos, se han reportado mayor ganancia de peso, mayor contenido de grasa, hiperleptinemia y resistencia a la insulina (Vogt et al. 2014).

Por otra parte también se ha encontrado que el consumo materno de una HFD disminuye la expresión de la hormona liberadora de tirotropina (THR), que es un blanco de neuropéptidos anorexigénicos; y reducción en la densidad de las fibras neuronales del núcleo arqueado (ARH), área involucrada en la regulación neuroendócrina y autónoma de la homeostasis energética (Vogt et al. 2014).

Por lo cual la sobrenutrición materna compuesta mayoritariamente de componentes lipídicos durante la lactancia es un factor de riesgo para el desarrollo de obesidad y sus complicaciones.

2.2.3.1 Resistencia a la insulina

La resistencia a la insulina es la disminución en la respuesta a la acción de la insulina en las células blanco de la hormona, como las del tejido adiposo, el músculo y el hígado. La obesidad o acumulación excesiva de tejido adiposo, resulta en un estado de inflamación crónica de bajo grado, así como en la liberación excesiva de ácidos grasos a la circulación (lipotoxicidad) (Sun et al. 2011, Boucher et al. 2014). El exceso de citocinas proinflamatorias como TNF-α, IL-6, IL-1β y la lipotoxicidad, la hiperglicemia, el estrés oxidativo, así como la disfunción mitocondrial y el estrés del retículo endoplásmico (Fig. 4) inducen resistencia a la insulina por múltiples mecanismos moleculares, dentro de los que

destaca la activación de cinasas que fosforilan residuos de serina (Ser) y de treonina (Thr). Este tipo de fosforilaciones reducen la activación del receptor de insulina (IR), del sustrato del receptor de insulina (IRS-1), y de otras moléculas río abajo en la vía de señal de la hormona, dando como resultado el desarrollo de resistencia a la insulina (Rotter et al. 2003, Zhang et al. 2008).

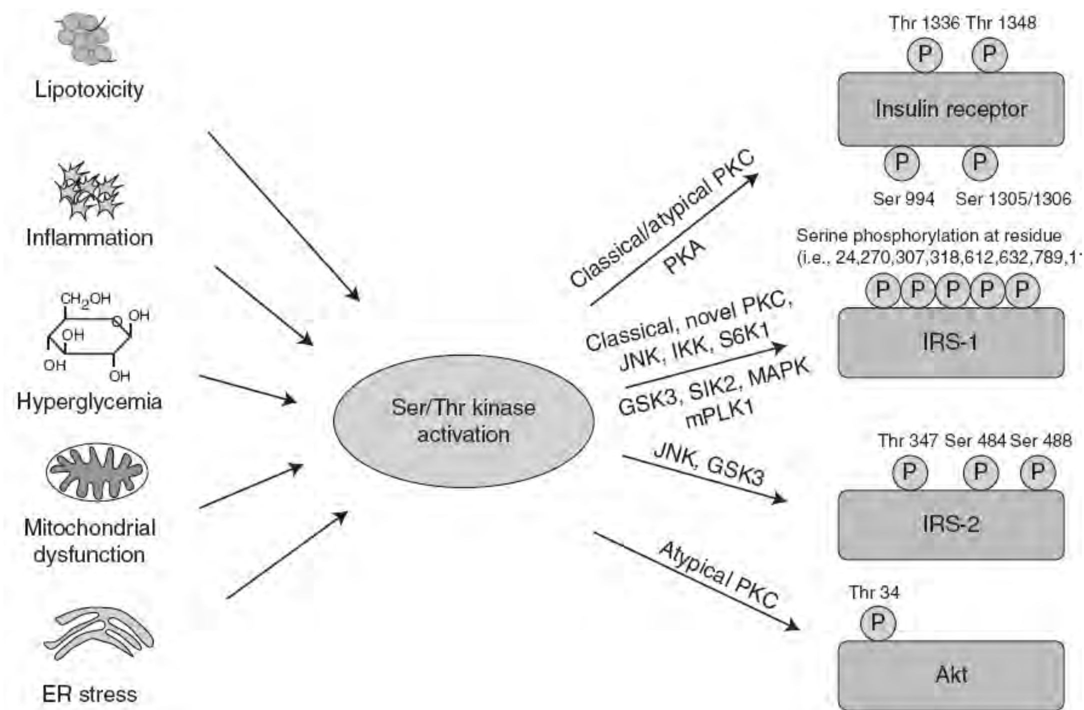

Fig. 4 Principales vías promotoras de resistencia a la insulina (Boucher et al. 2014).

-Lipotoxicidad

Una de las características de la obesidad es la acumulación de lípidos en tejidos no adiposos, como el hígado, el cual además juega un papel muy importante en el metabolismo, dando lugar al desarrollo del denominado hígado graso no alcohólico (Koonen et al. 2007).

Así mismo, el incremento en los niveles de ácidos grasos en el suero observados durante la obesidad inducen la fosforilación en residuos de Ser-307 del IRS-1 a través de las quinasas JNK, IKK y PKC (Schenk et al. 2008). Otro componente lipídico que promueve el desarrollo de resistencia a la insulina a través de la activación de la quinasa JNK y de la vía de NF-κB e induce estrés del retículo endoplásmico es el palmitato (Shi et al. 2006).

-Inflamación

Durante el desarrollo de la obesidad se presenta un estado de inflamación crónica de bajo grado, el cual está directamente asociado con la resistencia a la insulina. Específicamente la expansión del tejido adiposo derivada de una carga energética excesiva, genera la infiltración de macrófagos, produciendo un incremento en la secreción de citocinas proinflamatorias como TNF-α, IL1β, IL6, entre otras, las cuales a su vez favorecen la fosforilación del IRS-1 en residuos de Ser/Thr; además se ha reportado que el incremento en la secreción de dichas citocinas reducen la expresión del propio IRS-1, del transportador de glucosa GLUT-4 y de PPARγ (Hirosumi et al. 2002, Kanda et al. 2006, Jager et al. 2007).

-Hiperglicemia

Una de las principales alteraciones que ocurren durante el desarrollo de sobrepeso y obesidad derivada de un exceso en la ingesta calórica es la elevación de los niveles de glucosa sanguínea o hiperglicemia, la cual puede alterar la acción de la insulina en los tejidos metabólicos (Zisman et al. 2000, Kim et al. 2001) e inducir resistencia a la insulina a través de diversos mecanismos como el estrés oxidativo (Evans et al. 2005). Así mismo, la glucosa es uno de los principales sustratos para la formación de los productos de glicosilación avanzada, los cuales incrementan la fosforilación del IRS-1 en residuos de Ser-307 (Riboulet-Chavey et al. 2006). Por otra parte la hiperglicemia incrementa los niveles de fructosa a través de la vía de los polioles, incrementando el sustrato para la síntesis de glucosamina-6-fosfato, la cual promueve la resistencia a la insulina a través de la producción de la UDP-N-acetilglucosamina que es el sustrato para la enzima O-GlcNAc transferasa que cataliza su adición en los residuos de serina y treonina de los IRS (Patti et al. 1999). Por otra parte, los niveles elevados de glucosa incrementan la síntesis de diacilgliceroles, los cuales activan la vía de quinasas PKC, las cuales promueven la fosforilación en residuos de serina del IRS-1 (Miele et al. 2003).

-Estrés oxidativo y disfunción mitocondrial

El exceso de macronutrientes provenientes de la dieta, como la glucosa y los ácidos grasos libres son oxidados a través de la glucólisis y de la β-oxidación, para entrar en el ciclo de Krebs, incrementando el flujo de NADH y $FADH_2$ en la cadena respiratoria mitocondrial, lo que incrementa la producción del ion superóxido, dando lugar a mayor producción de especies reactivas de oxígeno (ROS), además también se produce una reducción en la expresión de enzimas antioxidantes (Fig. 5). El desbalance entre la producción de ROS y la defensa de los sistemas

antioxidantes produce estrés oxidativo. En condiciones de obesidad, se ha observado que el tejido adiposo es la mayor fuente de especies reactivas de oxígeno, las cuales son liberadas a la circulación afectando el balance oxidativo de todo el organismo (Evans et al. 2005). Las ROS estimulan vías de quinasas como la de NFκB, JNK y MAPK, promoviendo fosforilaciones en residuos de Ser en proteínas clave de la cascada de señalización de la insulina, promoviendo por tanto el desarrollo de resistencia a la insulina (Dokken et al. 2008).

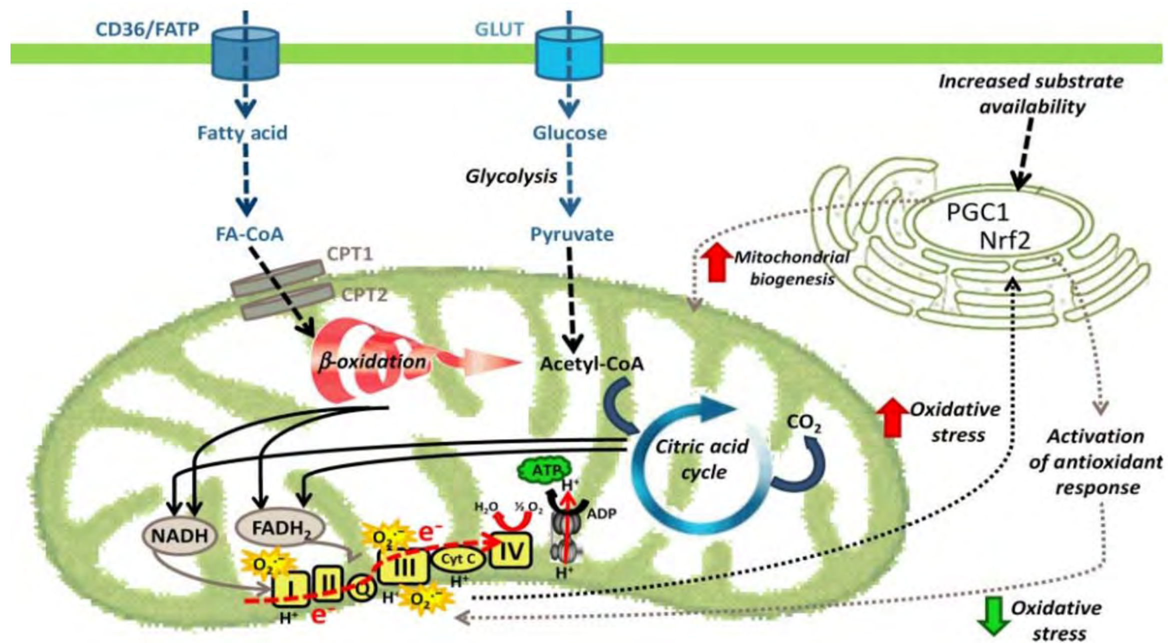

Fig. 5 Disfunción mitocondrial derivada de un exceso de macronutrientes (Montgomery et al. 2015).

-Estrés del retículo endoplásmico

Los ribosomas liberan las proteínas sintetizadas hacia el retículo endoplásmico, en donde proteínas chaperonas y foldasas realizan las modificaciones postransduccionales y el plegamiento de las proteínas. El estrés del retículo endoplásmico es un proceso que ocurre cuando existe acumulación de proteínas mal plegadas, es decir hay daños en su conformación, en su estructura, maduración y por lo tanto en su funcionamiento, de ahí que sea denominada respuesta a proteínas desplegadas (unfolded protein response, UPR). El propósito de la UPR es recuperar el funcionamiento de la célula deteniendo la traducción de proteínas y activando mecanismos para el plegamiento de las proteínas, y en último término induciendo la muerte celular programada (apoptosis) (Boden 2009).

Las vías involucradas en la respuesta a proteínas desplegadas del retículo endoplásmico son activadas en condiciones de obesidad, y se ha encontrado que en tejido adiposo e hígado se activan las quinasas JNK e IKK, estimulando la fosforilación en Ser-307 del sustrato del receptor de insulina (Ozcan et al. 2004, Hotamisligil 2010), favoreciendo la resistencia a la insulina.

2.2.4 Efectos del consumo de una dieta alta en grasa durante la lactancia sobre la funcionalidad de la glándula mamaria y la composición de la leche

2.2.4.1 Funcionalidad de la glándula mamaria

Se ha reportado que el consumo de dietas altas en grasa durante la lactancia, promueve el desarrollo de alteraciones en la estructura de la glándula mamaria, dentro de las que destaca la presencia de menor área parenquimal, es decir el tejido funcional mamario. Las principales alteraciones en la GM se deben al desarrollo anormal en la red alveolar que constituye parte fundamental para el funcionamiento de la glándula mamaria y/o a una reducción en su diferenciación debido a la presencia exacerbada de tejido adiposo en la glándula mamaria (Flint et al. 2005, Hernandez et al. 2012).

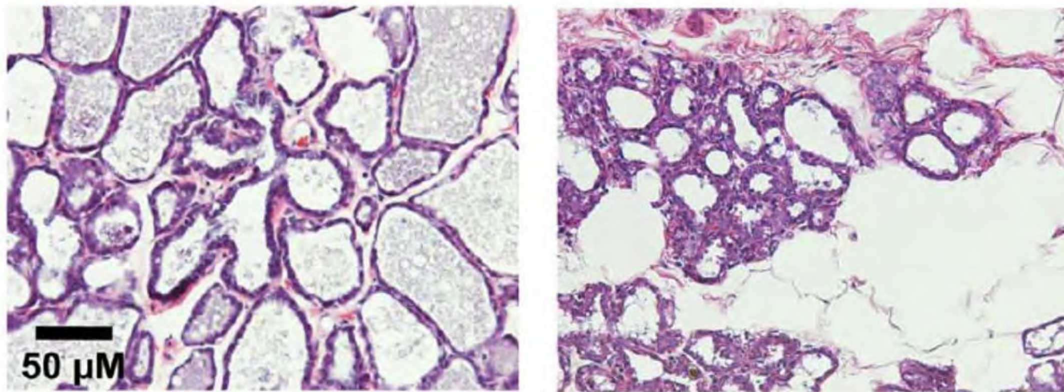

Fig. 6 Secciones histológicas de glándula mamaria de ratas lactantes en dieta control (izquierda) y en dieta alta en grasa (derecha) (Hernandez et al. 2012).

Específicamente se ha encontrado que el consumo de dietas altas en grasa promueve el desarrollo anormal de la GM durante la lactancia, a través de una reducción en los alveolos secretores de leche (Hernandez et al. 2012). Además, se ha observado un incremento significativo en la TPH-1, que es la enzima limitante para la síntesis de 5-HT (5-hidroxitriptamina o serotonina) que participa en el proceso de involución de la glándula mamaria. Es conocido que la 5-HT acelera la involución en respuesta a la disminución en el tránsito de la leche, uniéndose a su receptor (HTR7) y favoreciendo el rompimiento de las uniones

adherentes, disminuyendo los niveles de las proteínas de las uniones y disminuyendo la resistencia transepitelial (Stull et al. 2007, Pai et al. 2008). Así mismo también se ha reportado mayor expresión de citocinas proinflamatorias como TNF-α e IL-6 en la GM en condiciones de obesidad.

Por otra parte, la obesidad durante la lactancia puede generar un exceso en la acumulación de triglicéridos (TAG) mediante el incremento en la síntesis de leptina, citocinas proinflamatorias como el factor de necrosis tumoral α (TNF-α), e interleucina 6 (IL-6) por el tejido adiposo. Así mismo, el incremento en el tamaño de adipocitos y la producción de TNF-α, favorecen respuestas lipolíticas, que puede incrementar el flujo de ácidos grasos no esterificados (AGNEs) a la circulación, los cuales a su vez pueden promover la acumulación y disponibilidad de TAG en tejidos como el hígado y la glándula mamaria (Vernon 2005).

2.2.4.2 Alteraciones en la composición de la leche

El consumo de dietas altas en grasa durante la lactancia disminuye la expresión de genes relacionados con la síntesis de proteínas de la leche (caseína y proteína ácida de la leche), con la síntesis de lactosa (α-lactoalbúmina) y con la síntesis de lípidos (Acetil-CoA carboxilasa) (Flint et al. 2005, Hernandez et al. 2012).

Dentro de las principales alteraciones en la composición de la leche materna por una ingesta hipercalórica durante la lactancia, se encuentra la producción de lípidos, los cuales son la principal fuente de energía para el lactante, además de proveer los ácidos grasos necesarios para la síntesis de membranas celulares y el desarrollo del Sistema Nervioso Central (Zhao et al. 2009, Guesnet et al. 2011).

Por otra parte, se ha reportado que en condiciones de obesidad, la leche materna presenta un incremento significativo en los niveles de lípidos, específicamente en los ácidos grasos de cadena larga, mientras que los de cadena media se ven reducidos; así mismo, los niveles de proteína también se reducen (Rolls et al. 1986). De igual manera se ha reportado que el consumo de dietas altas en grasa resulta en una disminución en la leche materna de los ácidos grasos poliinsaturados eicosapentaenoico (EPA) y docosahexaenoico (DHA) y a su vez en un incremento en el contenido total de grasa, ácidos grasos libres y monosaturados (Bautista et al. 2016).

En los últimos años ha cambiado el concepto sobre que la leche materna, independientemente del estatus metabólico y nutricional de la madre, era el mejor alimento para el infante, debido a que se ha encontrado que el consumo de dietas hipercalóricas, promueve la producción de una leche inflamatoria, es decir, con

altos contenidos de ácidos grasos saturados de cadena larga; lo que se ha asociado con la acumulación de ceramidas e inflamación en las crías lactantes (Du et al. 2012).

También se han reportado alteraciones en los niveles de hormonas de la leche, las cuales ejercen acciones sobre procesos metabólicos, dentro de los que destaca la regulación de la ingesta, por lo tanto su concentración en la leche materna es fundamental para la prevención y control de alteraciones metabólicas en el lactante (Badillo-Suarez et al. 2017).

En relación a esto se han encontrado niveles elevados de leptina en la leche de madres alimentadas con una dieta alta en grasa, lo cual es relevante dado que la leptina regula positivamente la saciedad en una edad temprana, influenciando la programación nutricional y el balance energético durante la infancia y la vida adulta (Bautista et al. 2016, Badillo-Suarez et al. 2017).

2.3 Composición de la leche materna

Los componentes de la leche materna provienen de tres fuentes: 1) síntesis de novo en los lactocitos de la glándula mamaria, 2) las reservas de la madre y 3) la dieta materna; y varían entre mujeres y a lo largo de la lactancia. Así mismo, la leche materna va cambiando de manera dinámica en respuesta a las necesidades y características del lactante, como su edad, estado de salud, tiempo de succión, entre otros (Ballard et al. 2013).

La leche materna madura (4-6 semanas postparto) tiene un contenido energético de 650 a 700 cal/mL, de las cuales las grasas proporcionan la mayor parte de calorías. En relación a las macromoléculas, contiene aproximadamente 67-78 mg/mL de lactosa, 9-12 mg/mL de proteína y 32-36 mg/dL de grasa (Ballard et al. 2013).

De los carbohidratos, el componente más importante es la lactosa, y se sabe que el contenido de lactosa es proporcional al volumen de leche producido (Nommsen et al. 1991). Así mismo, la leche materna también contiene oligosacáridos, compuestos que en los últimos años han sido relacionados con la prevención de obesidad y resistencia a la insulina, a través de la protección de la microbiota intestinal y la regulación de la permeabilidad (Hamilton et al. 2017). Como por ejemplo los HMO (human milk oligosacharides), los cuales se encuentran en una alta proporción en la leche materna humana y que de acuerdo con su estructura será la función que ejercerán. Entre sus características, destaca que pueden soportar las condiciones intestinales, sin modificar su estructura y por tanto su

función, convirtiéndose en prebióticos para la microbiota intestinal; también bloquean la adhesión de microorganismos patógenos a la superficie intestinal protegiendo contra el desarrollo de procesos infecciosos; y además participan en la regulación del sistema inmune del neonato (Jantscher-Krenn et al. 2012, Bode 2015).

Las principales proteínas presentes en la leche son: α-lactoalbúmina, caseína, lactoferrina, inmunoglobulina A, lisozima y albúmina (Lonnerdal 2004). Así mismo se ha reportado que la dieta de la madre no altera los niveles de proteína en la leche, sin embargo, se ha encontrado que la proteína de la leche materna se ve reducida conforme se incrementa el IMC de la madre (Nommsen et al. 1991).

El componente más variable de la leche es la grasa, se conoce que tanto la cantidad como el tipo de ácidos grasos varían entre la leche que es liberada al inicio de la succión y entre la leche del final. Así mismo, también varían de acuerdo con la hora del día, la duración de la lactancia, la dieta de la madre entre otros (Saarela et al. 2005, Kent et al. 2006). En relación a la alimentación de la madre, se ha reportado que el consumo de dietas altas en grasa y carbohidratos está asociado con menor contenido de ácidos grasos poliinsaturados de cadena larga, por ejemplo se ha encontrado que la leche materna de madres de los Estados Unidos tiene un bajo contenido del ácido docosahexaenoico (DHA) (Valentine et al. 2010).

La leche materna también contiene otros micronutrientes como vitaminas A, B1, B2, B6, B12, D; y minerales como yodo, calcio, sodio, hierro, cobre y zinc, los cuales están más biodisponibles en comparación con los minerales presentes en la leche de otras especies, lo que se debe a las uniones que hacen con otros componentes de la leche, tales como las proteínas y los glóbulos de grasa (Fransson et al. 1983, Lonnerdal 2004).

Por otro lado también se han identificado compuestos bioactivos o con características funcionales en la leche materna, es decir son aquellos componentes que afectan algún (os) proceso (s) biológico (s) en la madre y/o el infante, por lo cual pueden tener impacto sobre la función del organismo y proporcionar beneficios a la salud más allá de sus características nutricionales (Biesalski et al. 2009). Estos componentes pueden ser sintetizados en el epitelio mamario, de otras fuentes celulares que son transportadas hacia la leche y de las que provienen del suero y atraviesan la membrana del epitelio mediante sus receptores.

Algunos de los compuestos funcionales que contiene la leche materna son factores de crecimiento como el factor de crecimiento epidermal (EGF), el cual se encuentra en cantidades elevadas al inicio de la lactancia, y tiene la función de proteger la función intestinal del infante, mediante la inhibición de la apoptosis y manteniendo las uniones del epitelio intestinal (Dvorak et al. 2003, Khailova et al. 2009). Otro ejemplo es el factor de crecimiento de tipo insulina (IGF), se encuentra en niveles elevados en el calostro y se ha reportado que en niños alimentados con leche materna presentan niveles más elevados de IGF que niños que no lo son. El IGF se ha relacionado también con protección intestinal (Elmlinger et al. 2007). Por otro lado el factor de crecimiento endotelial vascular (VEGF), está presente en la leche materna, y en niveles más elevados en el calostro, y se ha sugerido que protege contra la retinopatía del prematuro (Loui et al. 2012). Otro componente bioactivo presente en cantidades elevadas es la eritropoyetina (EPO), que se ha relacionado con prevención de la anemia en bebés prematuros y además con menor riesgo de enterocolitis necrotizante en el lactante (Soubasi et al. 1995, Shiou et al. 2011).

La leche materna también contiene hormonas, las cuales son consideradas funcionales por sus efectos sobre la salud. Entre estas hormonas se encuentra la leptina, la cual puede provenir del epitelio mamario y/o también del suero sanguíneo. Se ha encontrado que la leptina puede regular la ingesta alimentaria a corto plazo y ejercer un efecto a largo plazo sobre el control del balance energético y de la ganancia de peso (Smith-Kirwin et al. 1998, Bonnet et al. 2002, Stocker et al. 2008). La adiponectina también está presente en la leche materna, alcanzando sus niveles más altos al inicio de la lactancia, para posteriormente ir disminuyendo. Los niveles de adiponectina en el suero de recién nacidos presentan una correlación positiva con el peso y talla al nacer, la adiposidad neonatal y los niveles de leptina en suero (Martin et al. 2006, Inami et al. 2007).

Así mismo, también se encuentran otras hormonas como resistina, grelina, FGF21, obestatina, nesfatina, apelina, las cuales en general se han relacionado con efectos sobre la regulación de la ingesta de alimento y del balance energético, sugiriendo que tienen un papel sobre el desarrollo metabólico del infante a corto y largo plazo (Çatlı et al. 2014). Mientras que en la leche materna se encuentran otras hormonas, como la PRL, la cual se ha asociado con menor riesgo de desarrollar obesidad infantil (Chirico et al. 2013) y ha mostrado efectos positivos en condiciones de obesidad durante la etapa adulta (Lemini et al. 2015, Ruiz-Herrera et al. 2017), sin embargo sus efectos en el metabolismo de crías lactantes no ha sido descrito.

2.3.1 Prolactina

2.3.1.1 Características estructurales y secreción

La prolactina (PRL) es sintetizada en las células lactotropas de la glándula pituitaria, las cuales aumentan su proliferación durante el embarazo y la lactancia (Scheithauer et al. 1990). Aunque la mayor parte de la PRL circulante proviene de la pituitaria, muchos otros tejidos extrapituitarios la producen, como la glándula mamaria, el útero, cerebro, próstata, linfocitos, piel y el tejido adiposo (éste último solo en humanos). La PRL pertenece a la familia de las hormonas lactogenoplacentarias, en las cuales se encuentran la hormona del crecimiento (GH) y el lactógeno placentario (PL) (Gillam et al. 2011).

La PRL humana está compuesta por 199 aminoácidos, de los cuales existe cierto grado de homología con otras especies, por ejemplo, la PRL de ratón tiene el 61% de homología, mientras que la PRL de rata es capaz de activar el receptor de PRL humano (Utama et al. 2006). Aproximadamente el 90% de la PRL presente en el suero es el monómero de 23 kDa, el restante corresponde a otros agregados monoméricos de la PRL con diferentes grados de glicosilación, fosforilación o tamaños (Gillam et al. 2011).

Los niveles basales de PRL circulante van incrementando gradualmente durante el transcurso del embarazo, hasta alcanzar niveles por encima de 200 ng/mL; la elevación de los niveles de PRL al final del embarazo, permite preparar al tejido mamario para dar inicio a la lactancia (Rigg et al. 1977). Posteriormente en las primeras 4 semanas postnatales, la PRL basal se encuentra elevada en el suero de las mujeres lactantes, derivado del estímulo de la succión ejercida por el neonato, siendo por tanto el principal estímulo para la liberación de PRL por la pituitaria. Los niveles de PRL se mantienen elevados a lo largo del periodo de la lactancia, sin embargo van disminuyendo conforme transcurre el periodo de la lactancia, como resultado de la disminución de los periodos de succión y de la introducción de otros alimentos en el infante (Johnston et al. 1986). Durante la lactancia, la PRL también es producida por la glándula mamaria, sin embargo no se ha definido claramente, si la PRL presente en la leche materna es sintetizada localmente o proviene de la periferia (Zinger et al. 2003).

2.3.1.2 Función de la PRL durante la lactancia

Dentro de las principales funciones de la PRL se encuentran la osmoregulación, participa en el crecimiento y desarrollo, tiene efectos metabólicos, ejerce acciones sobre la reproducción, entre otros (Grattan et al. 2008). Una de las funciones más

importantes de la PRL, es su acción fisiológica sobre la lactancia, participando en la diferenciación de la glándula mamaria, hasta en el mantenimiento de la producción de leche.

La PRL es fundamental para el desarrollo y diferenciación de la GM, así como para el mantenimiento de la lactancia, debido a que se conoce que ratones hembra deficientes para el receptor de prolactina son incapaces de lactar dado que presentan alteraciones en el proceso de desarrollo de la GM, mostrado por ausencia de las ramificaciones de los ductos mamarios, y no presentan alveolos a lo largo de los ductos de la glándula mamaria (Ormandy et al. 1997, Ormandy et al. 2003).

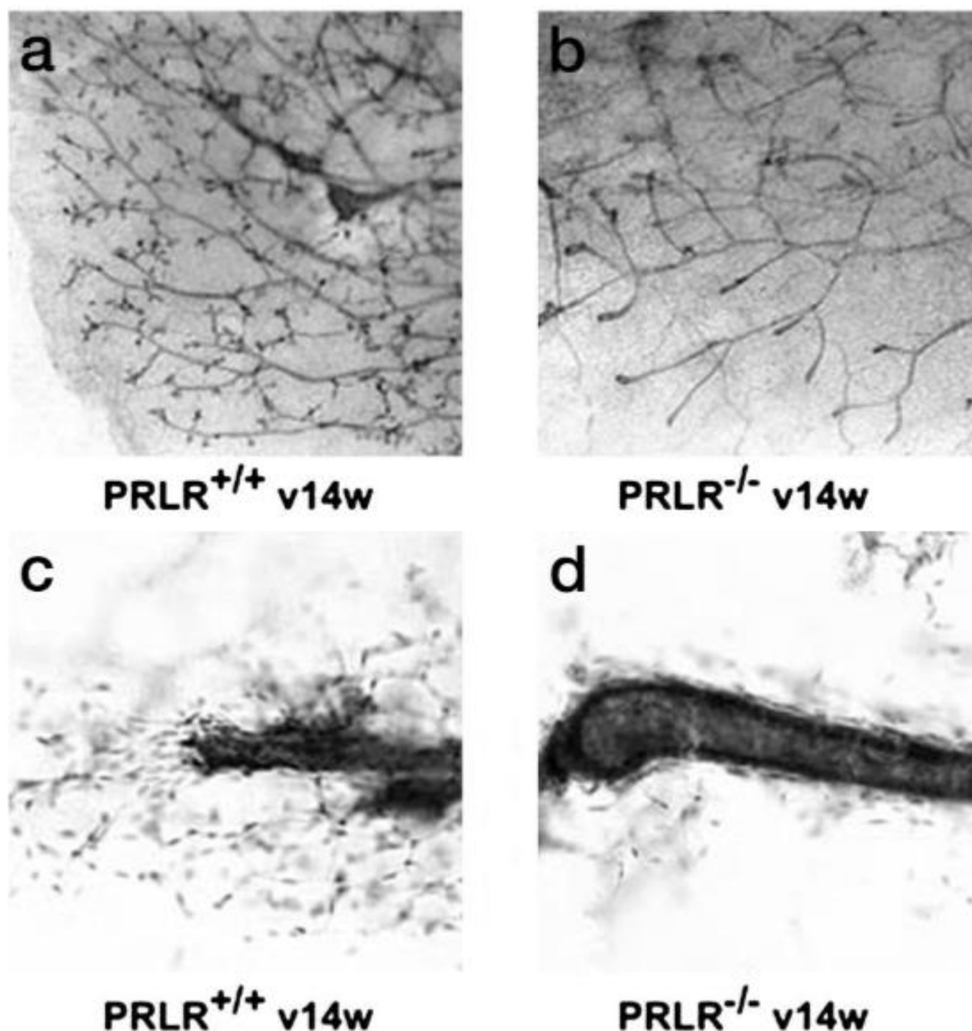

Fig. 7 Desarrollo de la glándula mamaria en hembras vírgenes a las 14 semanas de edad. Montaje completo de la ramificación ductal (A, B), y formación de brotes alveolares (C, D) (Ormandy et al. 2003).

El desarrollo de la glándula mamaria sucede en diferentes fases, los cuales se han estudiado ampliamente en modelos murinos. La primera fase consiste en la mamogénesis, al respecto se conoce que al nacimiento existe un sistema de pequeños ductos, que van creciendo lentamente hasta el inicio de la pubertad. En esta etapa ocurre un crecimiento exacerbado de los ductos mamarios, dando lugar a la formación del árbol ductal que ocupa todo el espacio de la GM. La diferenciación de las células epiteliales de los alveolos termina al final de la gestación, para dar lugar a la secreción de leche materna. Posteriormente comienza la lactogénesis o síntesis de leche, en este punto ocurre la activación secuencial de genes involucrados en la síntesis de proteínas de la leche, por lo cual la presencia de la PRL es fundamental, siendo esta última el principal factor regulatorio para la síntesis de dichas proteínas. De igual manera, es necesario el estrógeno y la progesterona para el crecimiento de los ductos mamarios y para la proliferación alveolar. Así mismo, una vez en la lactancia, la glándula muestra una elevación en los niveles de STAT5, lo que lo hace un candidato crítico para la terminación de la diferenciación del epitelio funcional de la GM. Posteriormente la propia PRL es la encargada de mantener la producción de leche o galactopoyesis a través de la succión del lactante, siendo el principal estímulo para la liberación de leche (Cowie et al. 1969, Hennighausen et al. 1997, Neville 1999). La PRL en conjunto con algunas otras hormonas, factores de crecimiento y citocinas, son esenciales para los cambios morfológicos que ocurren en el tejido mamario durante el embarazo y la lactancia (Neville 1999).

En el embarazo, el estradiol, la progesterona y el lactógeno placentario en conjunto con la PRL promueven la proliferación del epitelio lobuloalveolar. Posteriormente durante la lactancia, la progesterona, el estradiol y el lactógeno placentario disminuyen, dando lugar a un pico en los niveles de PRL, los cuales junto con las acciones de ligando de RANK y de IGF-2 inducen la formación de los acinos secretores (Brisken et al. 2002, Hovey et al. 2003). La presencia de los receptores de PRL son esenciales para llevar a cabo las funciones de la hormona (Brisken et al. 1999). Dentro de la vía de señalización de la PRL, algunos intermediarios son esenciales para mediar los efectos de la PRL sobre la morfogénesis alveolar como los factores de transcripción STAT5, SOCS-2 y Elf5 (Liu et al. 1997, Harris et al. 2006).

Durante la lactancia, la PRL regula la síntesis de proteínas de la leche como la β-caseína (Guyette et al. 1979), la lactoglobulina (Collet et al. 1991), la α-lactoalbúmina (Jagoda et al. 1991) y la proteína ácida de la leche (Hennighausen et al. 1991). Así mismo, también regula la síntesis de lactosa, promoviendo la expresión de la lactosa sintetasa, regulando positivamente la producción de leche. Adicionalmente, la PRL está involucrada en la síntesis de lípidos, a través de la

regulación de la expresión de enzimas involucradas en la lipogénesis, como la lipoproteína lipasa, la ácido graso sintasa y la acetil-CoA carboxilasa (Waters et al. 1988, Rudolph et al. 2011).

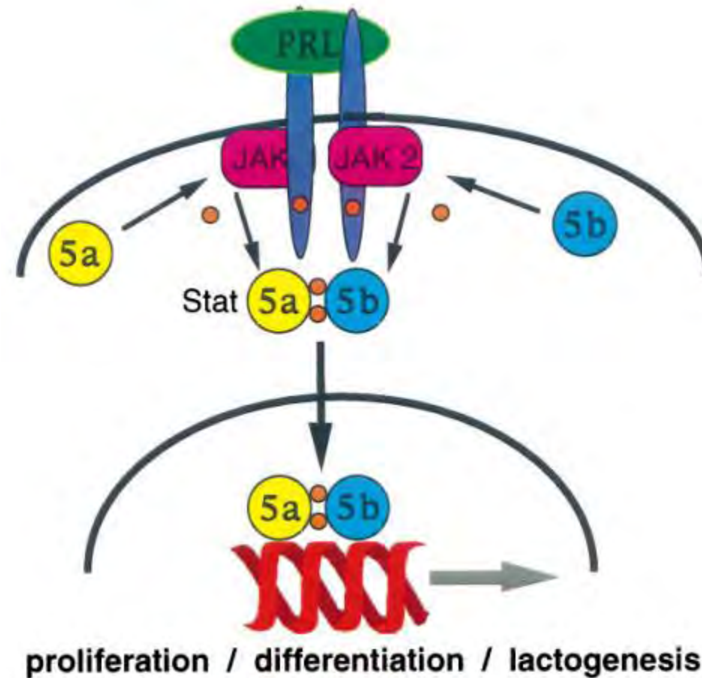

Fig. 8 Vía de señalización de la PRL en la GM (Hennighausen et al. 1997).

Interesantemente, la PRL está presente en la leche materna en grandes concentraciones y es capaz de llegar a la circulación de las crías alimentadas (Whitworth et al. 1978). Sin embargo, no se han descrito las funciones de la PRL presente en la leche materna sobre el metabolismo de las crías.

2.4 Papel metabólico de la prolactina

La PRL participa en la regulación de la homeostasis metabólica a través de diversas funciones sobre los principales tejidos metabólicos, como el hígado, tejido adiposo, páncreas, glándula mamaria, entre otros.

Recientemente la PRL ha sido identificada como promotora de la sensibilidad a la insulina, tanto en modelos animales como en humanos. Al respecto, estudios en niños y adultos, han mostrado que bajos niveles de PRL en la circulación se asocian con mayor prevalencia de obesidad, resistencia a la insulina, intolerancia a la glucosa, hígado graso, síndrome metabólico y diabetes tipo 2 (Balbach et al.

2013, Chirico et al. 2013, Wang et al. 2013, Wagner et al. 2014, Ruiz-Herrera et al. 2017, Zhang et al. 2018).

Así mismo, en trabajos previos con modelos animales de obesidad inducidos por dieta, se ha encontrado que ratas adultas con obesidad y resistencia a la insulina presentan niveles reducidos de PRL circulante. Mientras que al tratar a los animales con PRL, estos presentaron una mejora en la sensibilidad a la insulina, y en la disfunción del tejido adiposo, representado por una reducción en la hipertrofia de los adipocitos y en la inflamación de dicho tejido (Lemini et al. 2015, Ruiz-Herrera et al. 2017).

Por otra parte, se ha reportado en humanos y en modelos murinos, que la PRL regula la proliferación e inhibe la apoptosis de las células β pancreáticas, además de estimular la secreción de insulina (Freemark et al. 2002, Park et al. 2011). A través de su receptor media diferentes acciones, como la promoción de la adipogénesis e inhibición de la lipólisis en el tejido adiposo (Carre et al. 2014, Ben-Jonathan et al. 2015), además también se ha demostrado que promueve la sensibilidad a la insulina en el hígado, mediante la activación del factor de transcripción STAT5 que participa dentro de la vía de señalización de la PRL (Yu et al. 2013).

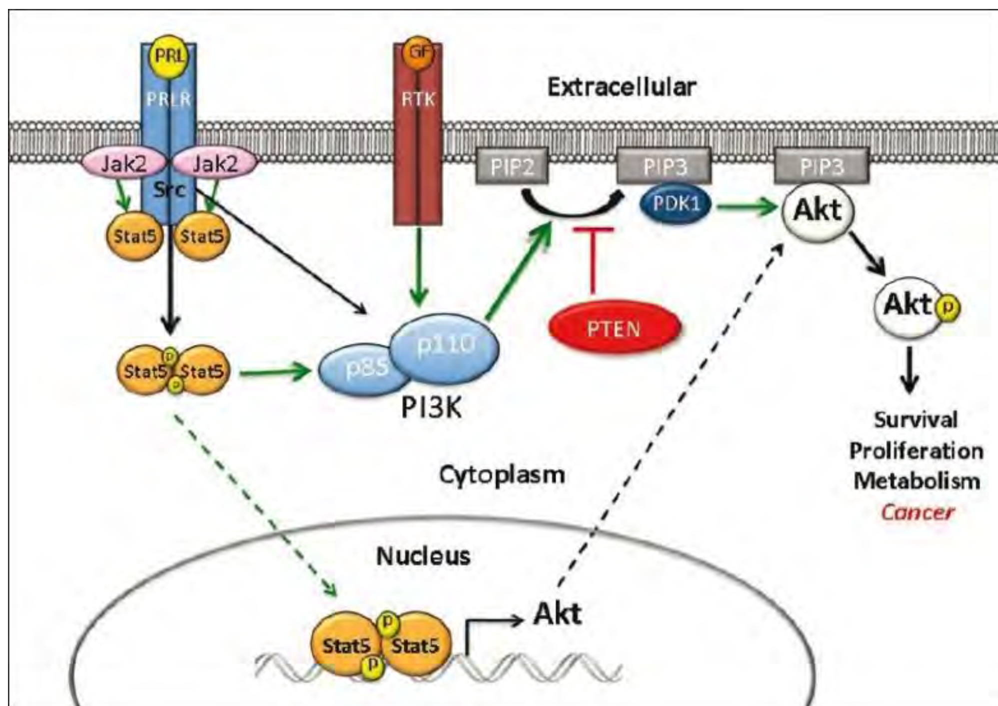

Fig. 9 Interacción de las vías Jak2/Stat5 y de PI3K/Akt en las células epiteliales de la glándula mamaria (Wagner et al. 2011).

3. JUSTIFICACIÓN

La lactancia materna es la alimentación ideal proporcionada por el seno materno durante los primeros años de vida, la cual contiene los nutrientes y componentes funcionales necesarios para el adecuado desarrollo del infante. Los niños alimentados con leche materna tienen un menor riesgo de presentar enfermedades infecciosas y menor prevalencia de desarrollar sobrepeso y obesidad. La composición de la leche materna está influenciada por la dieta, de ahí que la nutrición materna durante el periodo de lactancia sea un factor determinante para la salud metabólica de su progenie. Al respecto, el consumo materno de dietas hipercalóricas durante la lactancia genera daños metabólicos en las crías, los cuales pueden continuar hasta la etapa adulta. Dentro de los componentes funcionales de la leche materna se encuentran hormonas como la prolactina, la cual además de ser una de las principales hormonas reguladoras de la lactancia, se encuentra en altas concentraciones en la leche materna y puede llegar hasta la circulación de las crías amamantadas. Así mismo, la PRL ha demostrado ejercer efectos metabólicos benéficos en adultos en condiciones de obesidad, sin embargo, no han sido estudiados los efectos de la PRL sobre el metabolismo de la cría lactante, así como las alteraciones en los efectos de la hormona ante un ambiente obesogénico.

Considerando que actualmente la mayoría de las mujeres en edad reproductiva presentan obesidad o sobrepeso debido a el consumo de una dieta incorrecta, y que la nutrición materna ejerce una importante influencia sobre la salud metabólica de las crías, es fundamental identificar los factores presentes o ausentes en la leche que median los efectos adversos de una alimentación materna obesogénica en la salud de la progenie. Por lo anterior en este trabajo se evaluaron los efectos de la PRL proveniente de la leche materna sobre el metabolismo de las crías lactantes, las cuales fueron expuestas a condiciones metabólicamente adversas a través de la leche materna proveniente de madres alimentadas con una dieta control o una dieta alta en grasas durante la lactancia.

4. HIPÓTESIS

La PRL proveniente de la leche materna protege a las crías del desarrollo de obesidad y otras alteraciones metabólicas derivadas del consumo materno de una dieta alta en grasas durante la lactancia.

5. OBJETIVOS

5.1 Objetivo general

Investigar si el consumo materno de una dieta alta en grasas durante la lactancia altera los niveles e PRL en el suero y leche de la madre, y sí dichas alteraciones contribuyen con los efectos deletéreos derivados de una dieta alta en grasas sobre el metabolismo de las crías lactantes.

5.1.1 Objetivos específicos

5.1.1.1 Caracterizar el modelo del consumo materno de una dieta alta en grasas durante la lactancia sobre la ganancia de peso e ingesta de alimento en las madres, así como sobre la estructura y función de la glándula mamaria.

5.1.1.2 Determinar los efectos del consumo materno de una dieta alta en grasas durante la lactancia sobre los niveles de PRL en el suero materno y la leche, así como sobre la producción de leche.

5.1.1.3 Evaluar los efectos de la alimentación materna obesogénica durante la lactancia sobre el metabolismo de las crías.

5.1.1.4 Evaluar los efectos del tratamiento de restitución de PRL en ratas lactantes, en los niveles de la hormona en el suero y leche materna, la producción de leche y en las alteraciones de la estructura y función de la glándula mamaria derivadas de una dieta alta en grasas durante la lactancia.

5.1.1.5 Analizar los efectos del tratamiento de restitución de PRL tanto en madres alimentadas con una dieta alta en grasa como en sus crías, sobre parámetros metabólicos como adiposidad, resistencia a la insulina y acumulación de triglicéridos hepáticos en las crías.

5.1.1.6 Evaluar el mecanismo de acción de la PRL en el metabolismo de las crías tratadas con la hormona a través de la leche de las madres, o directamente por vía oral.

6. MATERIALES Y MÉTODOS

6.1 Modelo animal de obesidad durante la lactancia

Se emplearon 79 ratas Wistar hembras, en periodo de lactancia, con un peso corporal de entre 300–350 g, las cuales fueron proporcionadas por el bioterio del Instituto de Neurobiología de la UNAM.

Las ratas lactantes se mantuvieron a condiciones de temperatura ambiente 22-24°C y bajo el ciclo luz/oscuridad de 12h y con acceso libre a agua y alimento, correspondiente a una dieta control (CD) (2.91 kcal/g, de las cuales un 28% correspondió a proteínas, un 13.5% a grasas y un 58% a carbohidratos) (Laboratory Rodent Diet 5001; LabDiet, Richmond, IN, USA). Al día 1 de la lactancia (un día después de los nacimientos), se ajustaron las camadas a 8 crías por madre, además las ratas lactantes fueron divididas aleatoriamente en dos grupos de alimentación, unas continuaron con la CD, y el otro grupo recibió una dieta alta en grasas (HFD) (5.24 kcal/g, de las cuales un 20% correspondió a proteínas, un 60% a grasas y un 20% a carbohidratos) (OpenSource Diet D12492; Research Diets, New Brunswick, NJ, USA), dichas dietas fueron administradas durante los 21 días correspondientes al periodo de lactancia.

6.2 Tratamiento de restitución de PRL

6.2.1 Restitución de PRL en ratas lactantes: HFD+PRL

Al día 4 de la lactancia, las ratas lactantes alimentadas con una HFD se procesaron quirúrgicamente (fueron anestesiadas con éter, para una pronta y rápida recuperación) para la implantación subcutánea de mini-bombas osmóticas Alzet (Durect Corp., Cupertino, CA, USA) conteniendo una solución de PRL ovina (Sigma-Aldrich, St. Louis, MO, USA), la cual sería liberada en una dosis de 0.16 mg/kg/d a lo largo del periodo de lactancia, mientras que las madres en CD también fueron procesadas quirúrgicamente para asegurar tener las mismas condiciones entre todos los animales.

6.2.2 Restitución de PRL en crías lactantes: HFD+PRL-M y HFD+PRL-O

En el caso de las crías lactantes provenientes de madres alimentadas con una dieta alta en grasas recibieron el tratamiento de restitución de PRL a través de la leche de las madres que fueron tratadas con PRL proveniente de las mini-bombas osmóticas (HFD+PRL-M), o de manera directa a otro grupo de crías de madres del grupo HFD, se les administró PRL de manera oral (HFD+PRL-O) una vez al día en

una concentración similar a los niveles de PRL reportados en la leche de rata durante la lactancia (Bonomo et al. 2005, Bonomo et al. 2008), es decir comenzaron con una concentración de 1000 ng/mL en el día 4 de la lactancia y posteriormente fueron disminuyendo 50 ng/mL/d hasta llegar a 150 ng/mL en el día 21 de la lactancia. Para someter a todas las crías a la misma manipulación, el resto de las crías recibieron PBS oral a lo largo de la lactancia.

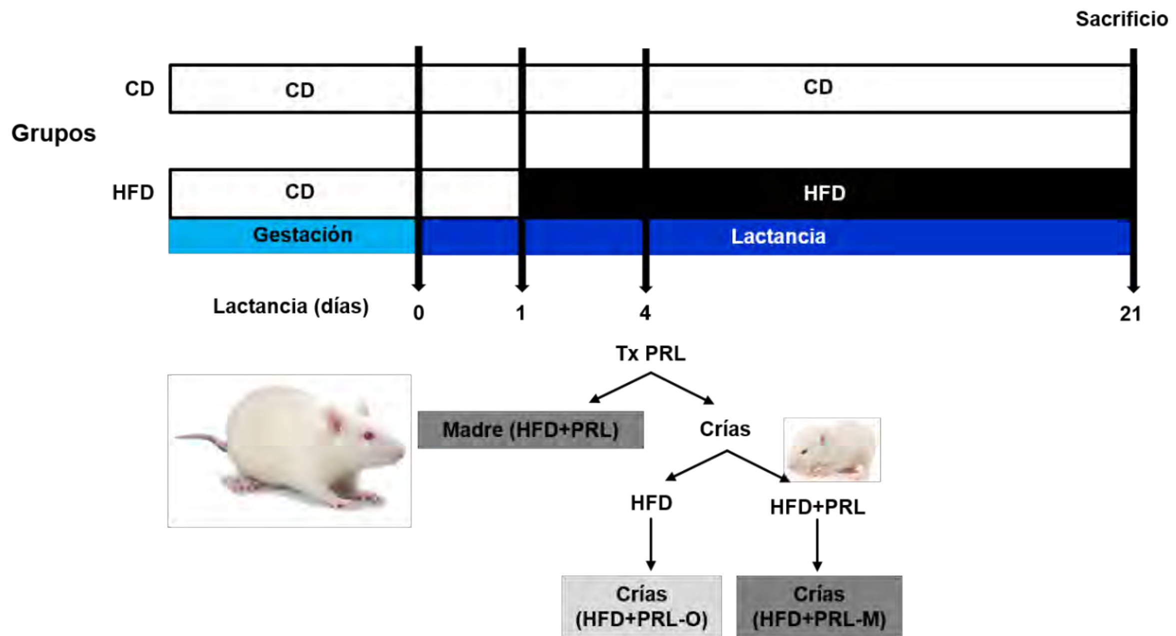

Fig 10. Estrategia experimental

6.3 Mediciones durante la lactancia

En los días 1, 3, 5, 10, 15 y 20 de la lactancia, fueron registrados los pesos de las madres y las crías. Así mismo, también se cuantificó la ingesta de alimento de las madres durante este periodo. En los días 7 y 21 se extrajo leche de las madres, y en los días 8 y 19 se realizó una estimación de la producción de leche.

En el día 19 de la lactancia se seleccionaron de 2 a 4 crías por madre para la realización de un ensayo de tolerancia a la insulina en las crías. Para evitar alteraciones en los niveles de PRL derivados de una reacción al estrés al momento del sacrificio, una semana antes al día final de la lactancia, los animales fueron manipulados diariamente bajo las mismas condiciones de aclimatación. Al día 21 de la lactancia los animales fueron sacrificados por inhalación con CO_2, para posteriormente obtener una muestra de sangre por decapitación, posteriormente se centrifugó para la obtención de suero, el cual fue dividido en alícuotas y se congeló y almacenó a -70°C para las determinaciones bioquímicas

posteriores. Así mismo, se extrajo el tejido adiposo visceral (Vis) y subcutáneo (Sc), hígado, músculo, páncreas, hipófisis, cerebro y glándula mamaria (GM) (solo para las madres), dichos tejidos se congelaron en nitrógeno líquido y se almacenaron a -70°C para los análisis correspondientes.

6.4 Determinaciones en suero y leche

Se cuantificaron los niveles de prolactina endógena (PRL de rata) mediante un ensayo de µELISA (Guillou et al. 2015) tanto en el suero de crías y madres, así como en la leche materna. Se utilizó una dilución de 1:50 para el suero de las madres tanto al día 7 como 21 de la lactancia. Para la leche se usó una dilución 1:500 y 1: 300 para el día 7 y 21 respectivamente.

Para medir los niveles de la PRL ovina (utilizada en los tratamientos de PRL en las madres y crías), se utilizó el Bioensayo celular Nb2 (Tanaka et al. 1980), basado en la respuesta proliferativa de las células de linfoma Nb2 a la PRL. En este ensayo se utilizó un anticuerpo específico de PRL anti-ovina (National Hormone and Peptide Programm C-3581016789; Research Resource Identifier RRIDAB_2629483). Los niveles de PRL ovina fueron obtenido mediante la diferencia entre la bioactividad obtenida en presencia o no de PRL ovina en el suero y leche de los animales tratados con la hormona.

Así mismo también se cuantificaron los niveles de insulina mediante un kit de ELISA (#90060, Crystal Chem Inc, IL, USA). Mientras que los niveles de triglicéridos en el hígado fueron determinados mediante un ensayo colorimétrico (#10010303 Cayman Chemical, Ann Arbor, MI, USA).

Con el propósito de cuantificar los niveles en suero de los lipolisacáridos de membrana de las bacterias como medida indirecta de la permeabilidad intestinal de las crías se utilizó el kit de ELISA Rat Lipopolysaccharides (LPS) (#CSB-E14247r, Cusabio, Maryland, USA).

6.4.1 Ensayo de tolerancia a la insulina

Este ensayo se realizó en las crías en el día 19 de la lactancia. Para esta determinación los animales fueron sometidos a un ayuno de 2 h, posteriormente se les midieron los niveles de glucosa mediante la obtención de sangre de la vena caudal a los tiempos 0, 15, 30, 60, 90 y 120 min, después de la administración de una inyección intraperitoneal de una solución de insulina a una dosis de 0.5 U/kg de peso corporal (HumulinR; EliLilly, Indianapolis, IN, USA).

6.4.2 Obtención y estimación de la producción de leche

Las muestras de leche al día 7 y 21 de la lactancia se obtuvieron de acuerdo al método descrito por DePeters *et* al., 2009 (DePeters et al. 2009). Para lo cual las crías fueron separadas de sus madres (para permitir la acumulación de leche en las glándulas mamarias) durante 4 horas, y 30 min antes de la recolección de leche, las ratas lactantes fueron inyectadas intraperitonealmente con oxitocina (Oxitopisa; Pisa Farmaceutica, Jal, Mexico) a una dosis de 8 UI/Kg, para inducir la eyección de leche. Posteriormente fueron anestesiadas con éter y se procedió a la extracción de la leche mediante un sistema de vacío. Las muestras de leche fueron almacenadas a -20° C para su posterior análisis.

Por otro lado la estimación de la producción de leche materna se realizó en los días 8 y 19 del periodo de lactancia de acuerdo al método descrito por Sampson *et al*., 1984 (Sampson et al. 1984). Las crías fueron pesadas (P1) al inicio de la estimación, después fueron separadas por 4 horas de sus madres. Transcurrido el tiempo se volvieron a pesar (P2) y se regresaron con sus madres para permitir que se alimentaran durante 1 h, y obtener su peso después del periodo de succión (P3). El rendimiento de la producción de la leche por hora fue calculado mediante la diferencia entre el peso de las crías después (P3) y antes de lactar (P2), al cual además se le añadió la pérdida de peso causada por los procesos metabólicos en las crías durante el periodo de experimentación (P2-P1)/4:

$$\text{Estimación de la producción de leche} = (P3 - P2) + \left(\frac{P2 - P1}{4}\right)$$

6.5 Histología de GM, Vis, Sc e hígado

La glándula mamaria (GM) de las ratas lactantes, el tejido adiposo visceral (Vis) y subcutáneo (Sc), así como el hígado de las crías fueron fijados en formalina al 10%, para después ser deshidratados y fijados en parafina. Se realizaron cortes de 5 µm para la GM, de 7 µm para el Vis y Sc y de 3 µm para el hígado; posteriormente fueron teñidos con hematoxilina y eosina.

En la GM se cuantificó el área parenquimal y el área adiposa en la GM, para lo cual se analizaron 9 campos de la glándula mamaria de cada animal. Mientras que para el Vis y Sc se calculó el área promedio de los adipocitos a partir de 9 campos por cría, y además se calculó el número relativo de adipocitos dividiendo el peso del tejido adiposo de cada animal, entre el promedio del área de los adipocitos. En el hígado se obtuvieron imágenes representativas de cada grupo con la finalidad

de observar su estructura y la presencia de gotas lipídicas. El análisis de las imágenes se realizó con el programa ImageJ (NIH).

6.6 Expresión de genes relacionados con la función de la GM por RT-PCR

Mediante la reacción en cadena de la polimerasa en tiempo real (RT-PCR) se determinó la expresión de genes relacionados con la función de la glándula mamaria al día 21 de la lactancia. Para lo cual se extrajo el ARN total de la GM usando TRIzol (#15596018, Invitrogen, Carlsbad, CA), después se realizó la síntesis de la cadena de ADN complementario con el kit de alta capacidad de retrotranscripción (#4368814, Applied Biosystems, Foster City, CA, USA).

Los productos fueron detectados y cuantificados con la reacción de Maxima SYBR Green / ROX qPCR Master Mix (#K0221, Thermo Fisher Scientific, Waltham, MA, USA) en una reacción final de 10 µL conteniendo el templado y los oligos de cada uno de los genes evaluados a una concentración de 0.5 µM: Tnf, Tph1, Csn2, Lalba, and Prlr. La amplificación se realizó en un RT-PCR CFX96 (Bio-Rad Laboratories, Hercules, CA) usando un protocolo de 10 min de desnaturalización a 95°C, seguido de 35 ciclos de amplificación (10 seg a 95°C, 30 seg a la temperatura de alineación de los oligos, y 30 seg a 72°C). La expresión de los genes relacionados con la función de la GM fue normalizada con el gen casero CypA.

	Secuencias de los oligos utilizados		
Nombre	Sentido	Antisentido	Temperatura de alineación °C
CypA	TGGGGAGAAAGGATTTGGCT	TTTGCCATGGACAAGATGCC	60.2
Tnf	GGGCTTGTCACTCGAGTTTT	TGCCTCAGCCTCTTCTCATT	59.4
Tph1	ACACCTGCCACGAACTCTTA	AGTGCATGTCTGAGCTCACT	62.5
Csn2	TCTTGCAGTCCCATTCCACA	GCATCTGTTTGTGCTTGGGA	55.5
Lalba	CTGCCTTTCAAGCCACAGAG	TGTTCTCTGACTCGGGGAAC	63.7
Prlr	ATCTTCAACATGGCCATTAC	TTCTTCCTCTCCAGTCTCAA	56.2

Tabla 1. *CypA* (Ciclofilina A), Tnf (Factor de necrosis tumoral α), Tph1 (Triptófano hidroxilasa 1), Csn2 (β-caseína), Lalba (α-Lactoalbúmina) y Prlr (Receptor de PRL).

6.7 Western blot

Muestras de 200 mg de hígado fueron homogenizados con buffer de lisis (0.1 M Tris-HCl, 0.2 M EGTA, 0.2 M EDTA, 0.1 M Ortovanadato de sodio, 50 mM Fluoruro de sodio, 100 mM Pirofosfato de sodio, 250 mM sacarosa, pH 7.5), posteriormente se cuantificó la concentración de proteína con el método de Bradford (Bio-Rad Laboratories, Hercules, CA). Después 60 µg de proteína de hígado fueron separadas mediante una electroforesis en gel de poliacrilamida con dodecilsulfato de sodio (SDS/PAGE), posteriormente se transfirieron en una membrana de nitrocelulosa, y se incubaron toda la noche con el anticuerpo primario anti phospho-STAT5 en una dilución 1:1000 (#9351, Cell Signaling Technology, Inc., Danvers, MA, USA) y con el anti-STAT5 en una dilución 1:500 (#sc-1081; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Las membranas se lavaron con Tris/Tween-20 y se revelaron usando el anticuerpo secundario a una dilución 1:5000 del goat anti-rabbit conjugado con peroxidasa de rábano (1:5000) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). La densidad de las proteínas fue cuantificada mediante el programa Quantity One (Bio-Rad, Richmond, CA, USA).

6.8 Permeabilidad intestinal *in vivo*

Para determinar los efectos de la PRL sobre la permeabilidad intestinal de las crías en el día 12 y 21 de lactancia, se les administró de manera oral una solución de FITC-Dextran de 4kDa (FD4) (#46944, Sigma-Aldrich, MO, USA), en una dosis de 750 mg/Kg de peso corporal. Las crías fueron sacrificadas 4 h después de la administración del FD4, con la finalidad de asegurar el suficiente tiempo de tránsito a través del intestino. Una vez sacrificadas las crías, se recolectó el plasma para la medición de la concentración del FD4 en un fluorómetro (Ex 485 nm: Em 525 nm).

6.9 Análisis estadístico

El análisis estadístico de los datos se realizó utilizando el paquete estadístico Minitab 16 (Minitab Inc., PA, USA) y el GraphPad Prism 5 (GraphPad software, CA, USA). Los resultados se expresaron como la media ± el error estándar (EE). Así mismo, se realizó el análisis de varianza (ANOVA) correspondiente, con el fin de determinar los cambios estadísticamente significativos, y se realizó un análisis de comparación de medias aplicando la prueba de Tukey utilizando un intervalo de confianza del 95%.

7. RESULTADOS

7.1 Caracterización del consumo materno de una dieta alta en grasas durante la lactancia

7.1.1 Ganancia de peso e ingesta de alimento en las madres

Con el fin de evaluar los efectos de la PRL durante la lactancia en un modelo de alimentación obesogénica, a un grupo de ratas lactantes se les dio una dieta alta en grasas (HFD) o una dieta control (CD) a lo largo del periodo de lactancia. Así mismo, siguiendo nuestra hipótesis de que el consumo materno de una HFD durante la lactancia reduciría los niveles de PRL en el suero y la leche de la madre, las ratas lactantes alimentadas con la HFD fueron tratadas (HFD+PRL) o no (HFD) con PRL proveniente de mini-bombas osmóticas implantadas subcutáneamente en el día 4 de la lactancia. Para caracterizar el modelo, se realizaron las mediciones de algunos parámetros metabólicos en las madres.

Se encontró que el consumo materno de una HFD durante la lactancia no modifica el peso corporal en las madres a lo largo de la lactancia, en comparación con las madres en CD, esto también se puede observar en el área bajo la curva del peso corporal del día 1 al día 20 de la lactancia, en donde no se observan diferencias entre los grupos (Fig. 11A). Mientras que la ganancia de peso de las madres al día 20 de la lactancia respecto al día 1, fue menor en las madres en HFD en comparación con las madres en CD (Fig. 11B), lo que sugiere un mecanismo diferente de almacenamiento o utilización del exceso energético proveniente de la dieta. Este resultado es similar a lo reportado en otros trabajos en animales lactantes en donde usan modelos de obesidad a largo plazo (Rolls et al. 1980, Ferezou-Viala et al. 2007).

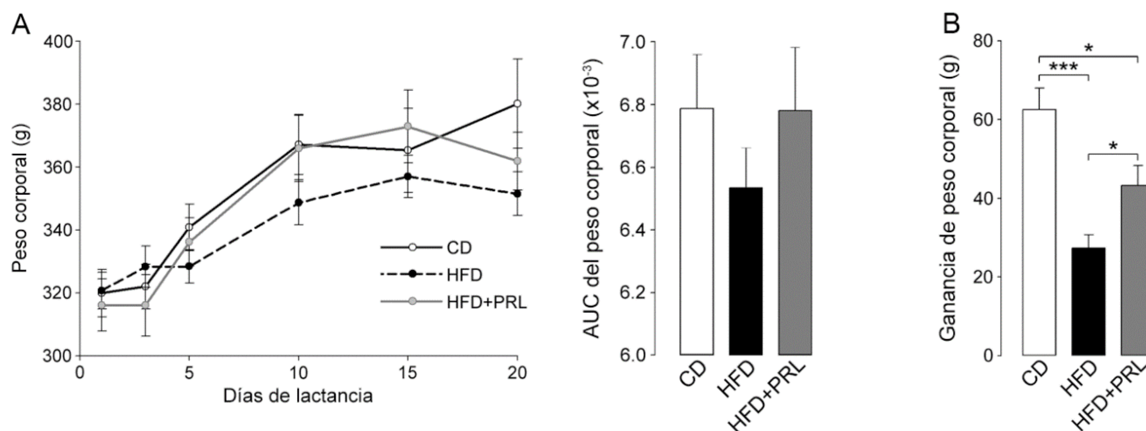

Fig. 11 Efectos del consumo materno de una HFD durante la lactancia sobre la ganancia de peso en ratas lactantes. Peso corporal y área bajo la curva (AUC) del peso corporal durante la lactancia (A), y ganancia de peso corporal entre el día 1 y 21de la lactancia (B) en ratas lactantes alimentadas con CD o HFD tratadas o no con PRL liberada a través de mini-bombas osmóticas (HFD+PRL) durante la lactancia (n=6–13). Los datos están representados por los promedios ± EE. *P<0.05, ***P<0.001. Las diferencias estadísticas significativas se determinaron con un ANOVA de una vía seguido de una prueba post hoc de Tukey.

Interesantemente, cuando las ratas sometidas al reto de la HFD, fueron tratadas con PRL, mostraron una curva de peso corporal muy similar a las madres en CD (Fig. 11A), así mismo se observa que presentaron mayor ganancia de peso en comparación con las ratas en HFD. Este resultado sugiere que la PRL participa en la regulación del gasto energético de la madre, por lo cual se determinó la ingesta de alimento y la ganancia de peso de la camada.

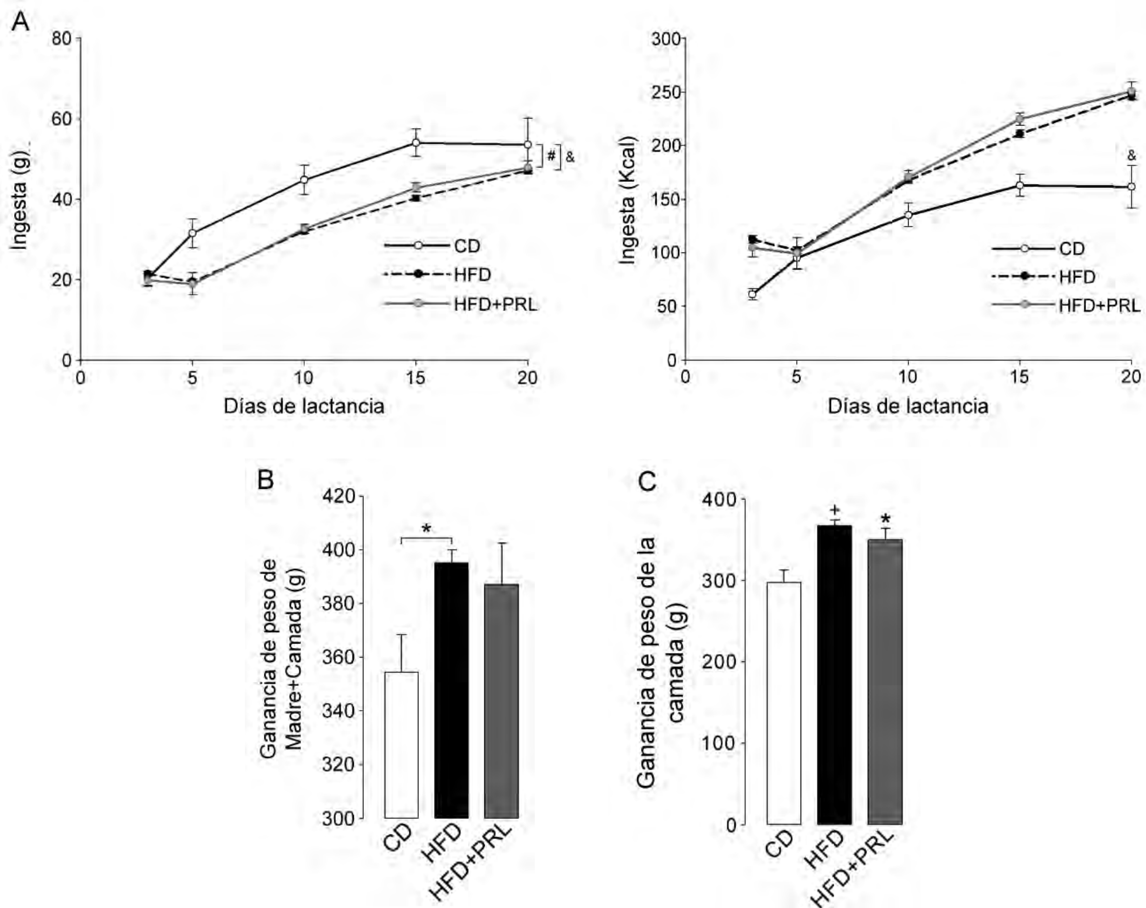

Fig. 12 Efectos del consumo materno de una HFD durante la lactancia sobre la ingesta de alimento en ratas lactantes. Ingesta de alimento en gramos y kilocalorías (A) en ratas lactantes alimentadas con CD o HFD tratadas o no con PRL liberada a través de mini-bombas osmóticas (HFD+PRL) durante la lactancia (n=6–13). Ganancia de peso

corporal entre el día 1 y 21 de la lactancia, del conjunto de la madre y su camada (B) y de la camada (C) (n=6-13 madres y camadas). Los datos están representados por los promedios ± EE. *P<0.05, **P<0.01, ***P<0.001, #P<0.01 vs. CD, &P<0.05 vs. CD. Las diferencias estadísticas significativas se determinaron con un ANOVA de una vía o por un ANOVA de medidas repetidas (A) seguido de una prueba post hoc de Tukey.

En cuanto a la ingesta de alimento, el consumo de una HFD independientemente del tratamiento con PRL, resultó en una disminución significativa de los gramos consumidos de alimento, sin embargo, tomando en cuenta el alto contenido calórico de la HFD, la ingesta calórica de las madres en HFD al finalizar la lactancia fue del 53% mayor en comparación con el grupo en CD (Fig. 12A). Este resultado resulta contradictorio si se considera que las madres en HFD mostraron menor ganancia de peso en comparación con las madres en CD; lo que sugiere dos posibilidades: la primera es que las ratas del grupo HFD tuvieran mayor gasto energético, y la segunda es que este exceso de calorías fuera transferido a la leche materna, por ejemplo, en forma de ácidos grasos.

Para determinar el destino del exceso energético que consumieron las madres en HFD, se determinó la ganancia de peso de las madres junto con sus crías (camada) (Fig. 12B) y se encontró que el grupo en HFD presentó 11.5% mayor ganancia de peso en comparación con las madres y crías del grupo CD. Mientras que las ratas lactantes tratadas con PRL (HFD+PRL) y sus crías no presentaron diferencias con ninguno de los otros grupos. Así mismo, también se evaluó el peso de la camada, en donde se encontró que las crías de las madres alimentadas con la HFD con o sin el tratamiento de PRL, presentaron mayor ganancia de peso al finalizar la lactancia (Fig. 12C). Este resultado muestra que el exceso de calorías ingeridas por las madres lactantes se acumula en la leche y ocasiona un mayor peso corporal en sus crías.

7.1.2 Niveles de PRL en suero y leche

Para evaluar sí el consumo materno de una HFD podría alterar los niveles de PRL en el suero de las madres y/o en la leche materna, se obtuvieron muestras de suero y leche en dos puntos del periodo de la lactancia, en el día 7 y en el día 21.

Se encontró que el consumo materno de una dieta alta en grasa no produce alteraciones significativas en los niveles de PRL en el suero materno (Fig. 13A) en comparación con las madres alimentadas con CD. Por otro lado, como se esperaba el grupo HFD+PRL presentó niveles más elevados de PRL en el suero en comparación con las ratas lactantes en CD y en HFD, tanto en el día 7 como en el día 21 de la lactancia (Fig. 13A).

En relación con los niveles de PRL en la leche materna se ha reportado que sus niveles se encuentran elevados en este fluido, en comparación con los niveles en el suero, específicamente están altamente concentrados al inicio de la lactancia y posteriormente va disminuyendo su concentración (Melo et al. 2009). En concordancia, los niveles de PRL en la leche que presentaron las ratas lactantes en condiciones control fueron de 576 ± 68 ng/ml al día 7 de lactancia y llegaron hasta 152 ± 22 ng/ml en el día 21, los cuales fueron de 4 a 6 veces más elevados que los niveles de PRL presentes en el suero (Fig. 13B).

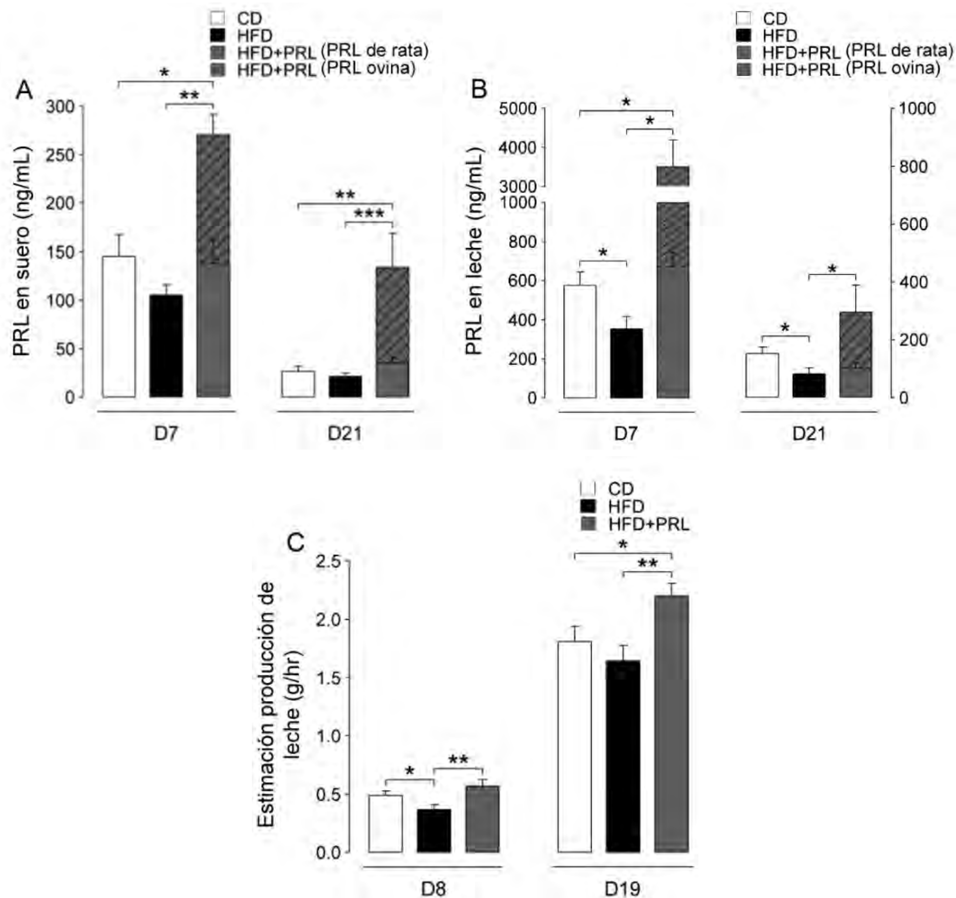

Fig. 13 El consumo materno de una dieta HFD durante la lactancia reduce los niveles de PRL en la leche. PRL en el suero (A) y PRL en la leche (B) evaluadas en el día 7 y 21 de la lactancia en ratas lactantes alimentadas con CD o HFD tratadas o no con PRL liberada a través de mini-bombas osmóticas (HFD+PRL) durante la lactancia (n=5-7). Estimación de la producción de leche (C) al día 8 y 19 de la lactancia (n=40-48). Los datos están representados por los promedios ± EE. *P<0.05, **P<0.01, ***P<0.001. Las diferencias estadísticas significativas se determinaron con un ANOVA de una vía seguido de una prueba post hoc de Tukey.

Interesantemente, el consumo materno de una dieta alta en grasa resultó en niveles reducidos de PRL en la leche tanto al día 7 de lactancia como al día 21, en comparación con las madres alimentadas con una dieta control. Cuando las madres fueron tratadas con PRL mediante las mini-bombas osmóticas, se generó un incremento de 6 veces en los niveles de PRL de la leche al día 7 de lactancia y de 2 veces al día 21, en comparación con lo observado en las madres en CD (Fig. 13B).

Así mismo, también se evaluó la producción de leche como una medida directa de la función de la PRL durante la lactancia (Neville et al. 2002), a través de la estimación de la ganancia de peso de las crías después de succionar durante una hora. Acorde con los niveles de PRL en la leche, las ratas alimentadas con HFD presentaron una reducción significativa en la producción de leche del 24% en comparación con las madres alimentadas con CD en la lactancia temprana (día 8), mientras que dicha reducción fue contrarrestada por el tratamiento con PRL en las madres. Al día 19 (lactancia tardía), las madres en HFD no presentaron alteraciones en la producción de leche, mientras que las madres tratadas con PRL (HFD+PRL) presentaron mayor producción de leche comparadas con los otros grupos (Fig. 13C). Estos resultados muestran que el consumo materno de una dieta alta en grasa durante la lactancia genera una disminución en la producción de leche, lo cual es revertido por el tratamiento con PRL. Este resultado sugiere daños en la función y/o estructura de la glándula mamaria (GM).

7.1.3 Estructura y función de la glándula mamaria

Para determinar si la reducción en los niveles de PRL de la leche se debían a alteraciones en la estructura y/o función de la GM, se analizó la morfología del tejido mamario mediante la tinción de hematoxilina y eosina, mientras que para la función se cuantificó la expresión de genes involucrados en la síntesis de leche como la α-lactoalbúmina (*Lalba*), en la síntesis de proteínas de la leche como la β-caseína (*Csn-2*) (Neville 1999) y en relación a la acción de la PRL, se midió su receptor (*Prlr*); así mismo también se cuantificó la expresión del factor de necrosis tumoral α (*Tnf*) (Piantoni et al. 2010), marcador de inflamación en procesos de involución, así como de la enzima triptófano hidroxilasa (*Tph1*) que participa en el proceso de involución de la GM (Matsuda et al. 2004, Hernandez et al. 2012).

El análisis de la estructura de la GM evidenció que el consumo materno de una HFD solo durante la lactancia, produjo mayor peso del tejido (Fig. 14A), así como el desarrollo de alteraciones en la morfología, representadas por un incremento en la cantidad de tejido adiposo (Fig. 14B) y una reducción del área parenquimal de la GM (Fig. 14C), así como una reducción significativa de la relación entre el área

parenquimal y el área adiposa (Fig. 14D), en comparación con la estructura presentada en las madres alimentadas con una CD, esto puede observarse claramente en las imágenes representativas de la GM de cada uno de los grupos (Fig. 14E). Por otra parte, las madres tratadas con PRL (HFD+PRL), mostraron un área parenquimal y adiposa similar a las madres en CD (Fig. 14 B,C), por lo tanto tuvieron una mejor relación entre estas dos áreas (Fig. 14D), resultados que de igual manera pueden observarse en las secciones de GM presentadas en la Fig. 14E.

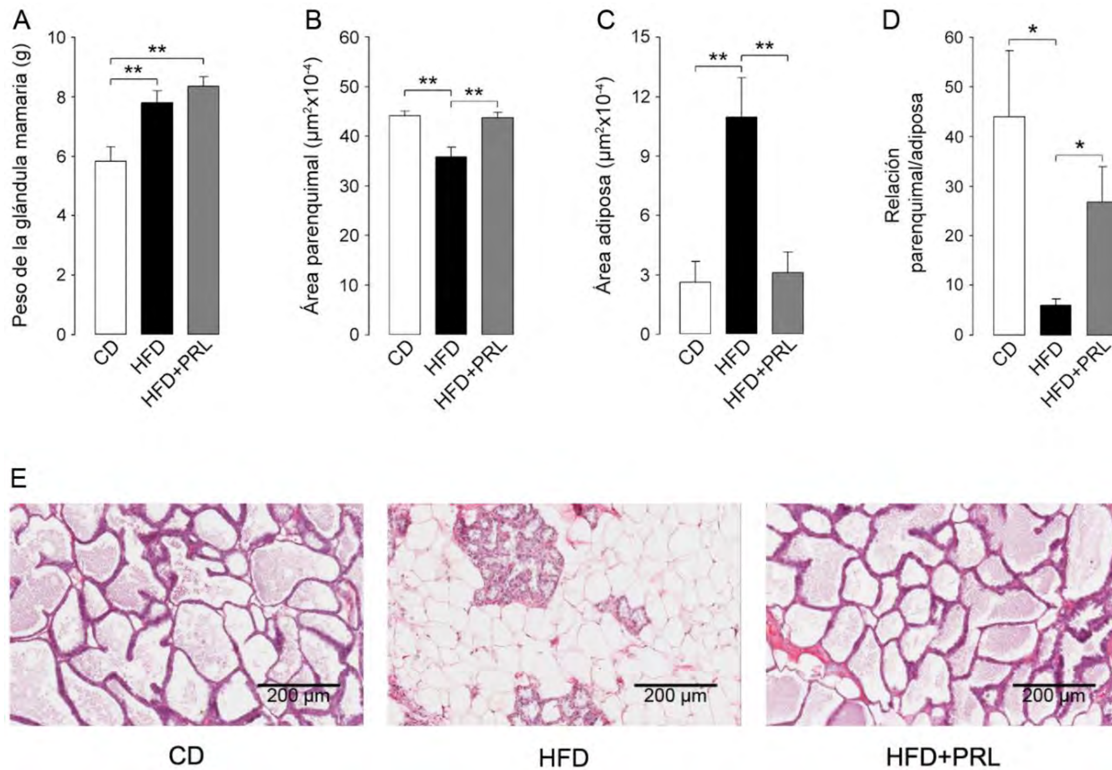

Fig. 14 El consumo materno de una HFD durante la lactancia altera la estructura de la GM, mientras que el tratamiento con PRL los previene. Peso de la GM (A), Área parenquimal (B), Área adiposa (C), Relación entre el área parenquimal y adiposa (D) y Secciones representativas de la GM teñidas con H&E (E). Todas las evaluaciones se realizaron al día 21 de la lactancia en ratas lactantes alimentadas con CD o HFD tratadas o no con PRL liberada a través de mini-bombas osmóticas (HFD+PRL) durante la lactancia (n=6-13). Los datos están representados por los promedios ± EE. *P<0.05, **P<0.01. Las diferencias estadísticas significativas se determinaron con un ANOVA de una vía seguido de una prueba post hoc de Tukey.

Así mismo, también se encontró que el consumo materno de una HFD durante la lactancia genera alteraciones en la funcionalidad del tejido mamario, dado que las ratas lactantes que recibieron una HFD exhibieron una expresión reducida de la α-

lactoalbúmina y de la β-caseína en comparación con las madres en CD, dichas proteínas de la leche están relacionadas con la cantidad de leche producida, dado que la α-lactoalbúmina se requiere para la formación de lactosa, la cual es un marcador del volumen de leche, y por otra parte la β-caseína promueve la síntesis de proteínas la leche (Neville 1999). Además, las madres en HFD presentaron una disminución significativa del receptor de PRL (*Prlr*) en la GM, lo que sugiere una reducción de las acciones de la PRL en el tejido mamario (Fig. 15A). Por otro lado, las madres del grupo en HFD tratadas con PRL, presentaron una normalización en la expresión de los genes relacionados con la función de la GM (Fig. 15A).

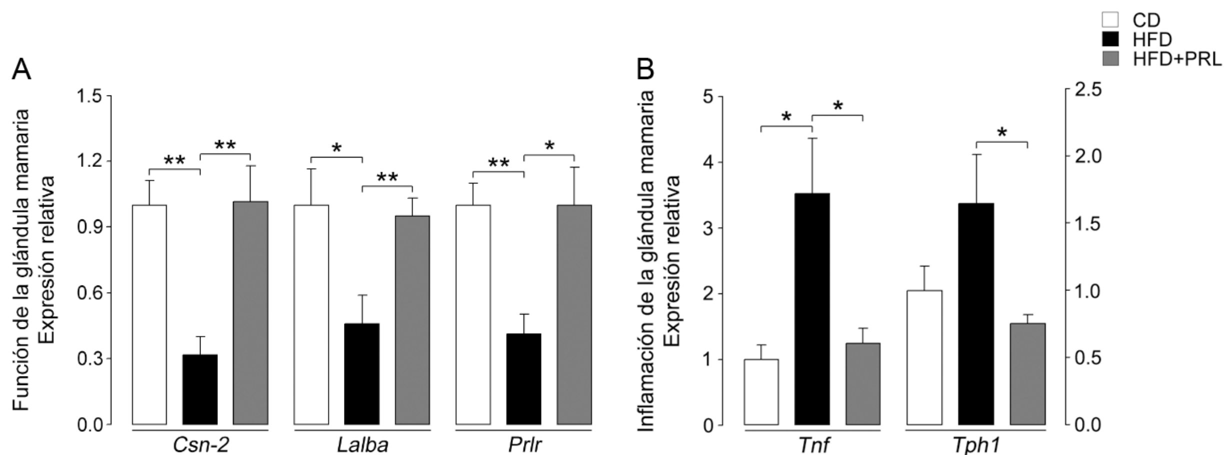

Fig. 15 El consumo materno de una HFD durante la lactancia altera la expresión de marcadores de función de la GM, mientras que el tratamiento con PRL previene dichos daños. Análisis por qPCR de la expresión de genes relacionados con la función de la GM, β-caseína (*Csn-2*), α-lactoalbúmina (*Lalba*) y el receptor de PRL (*Prlr*) (A), y con el proceso de involución de la GM, factor de necrosis tumoral α (*Tnf*) y la triptófano hidroxilasa 1 (*Tph1*) (B). Todas las evaluaciones se realizaron al día 21 de la lactancia en ratas lactantes alimentadas con CD o HFD tratadas o no con PRL liberada a través de mini-bombas osmóticas (HFD+PRL) durante la lactancia (n=6-11). Los datos están representados por los promedios ± EE. *P<0.05, **P<0.01. Las diferencias estadísticas significativas se determinaron con un ANOVA de una vía seguido de una prueba post hoc de Tukey.

Además, también se encontró que las ratas lactantes en HFD presentaron un incremento significativo en la expresión del factor de necrosis tumoral α (*Tnf*), y de la enzima limitante para la biosíntesis de serotonina (Fig. 15B), estos dos marcadores están involucrados en el proceso de involución, es decir el proceso por el cual la GM regresa a un estado no lactante (Stull et al. 2007, Pai et al. 2008). Mientras que las madres alimentadas con una HFD y tratadas con PRL, mostraron una expresión reducida, similar a los niveles de expresión observados

en las madres en CD, lo que sugiere un efecto protector de la PRL sobre la función de la GM ante el reto de una HFD (Fig. 15B).

Estos resultados concuerdan con lo reportado en modelos de obesidad a largo plazo, es decir en aquellos donde las madres son alimentadas con dietas altas en grasas desde antes del embarazo (Flint et al. 2005, Du et al. 2012, Hernandez et al. 2012), en donde se reportó que la disminución en la eficiencia del proceso de la lactancia es debido al desarrollo de resistencia a la prolactina en la GM (Turcksin et al. 2014, Buonfiglio et al. 2016), debido a una sobreactivación de la pSTAT5 que no puede ser estimulada aún más por PRL. Por lo cual estos resultados muestran que los trastornos en la estructura y función de la GM derivadas de una alimentación alta en grasas durante la lactancia, involucran alteraciones en las acciones de la PRL sobre la GM, las cuales son prevenidas por el tratamiento con PRL.

7.2 Evaluación de la función metabólica de la PRL en crías provenientes de madres alimentadas con una HFD durante la lactancia

7.2.1 Ganancia de peso corporal y adiposidad de las crías

Con la finalidad de determinar los efectos de la PRL sobre el metabolismo de crías provenientes de madres alimentadas con una dieta alta en grasa durante la lactancia, se evaluó su ganancia de peso corporal al finalizar la lactancia, así como su adiposidad visceral y subcutánea, además del tamaño y número de adipocitos en ambos tejidos de las crías.

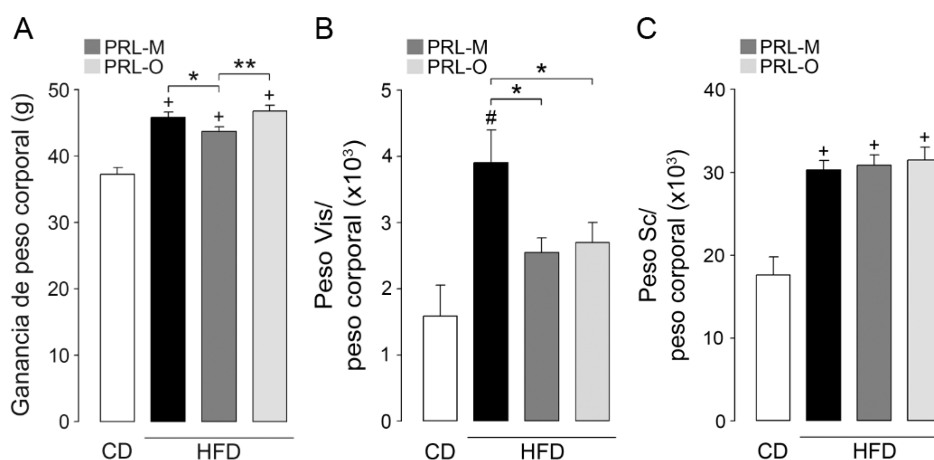

Fig. 16 El consumo materno de una HFD durante la lactancia, promueve el desarrollo de obesidad a través de una mayor ganancia de peso y adiposidad en las crías al destete, mientras que el tratamiento con PRL en las madres o por vía oral en

las crías previene dichas alteraciones. Ganancia de peso corporal (A), Peso del tejido adiposo visceral (Vis) (B) y subcutáneo (Sc) (C) normalizados con el peso corporal (n=11-20). Las evaluaciones se realizaron al día 21 de la lactancia en las crías de madres alimentadas con una CD o una HFD, y que recibieron o no el tratamiento materno de PRL (PRL-M) mediante mini-bombas osmóticas subcutáneas, o el tratamiento de PRL en las crías (PRL-O) vía oral durante la lactancia. Los datos están representados por los promedios ± EE. *P<0.05, **P<0.01, +P<0.001 vs CD, #P<0.01 vs CD. Las diferencias estadísticas significativas se determinaron con un ANOVA de una vía seguido de una prueba post hoc de Tukey.

Los resultados concuerdan con lo reportado en trabajos donde han observado que el consumo materno de una HFD durante la lactancia genera alteraciones metabólicas en sus crías (Sun et al. 2012, Vogt et al. 2014, Du et al. 2015, Liang et al. 2016). Esto debido a que se encontró que las crías de madres en HFD presentaron un 23% más ganancia corporal al finalizar la lactancia comparadas con las crías de madres en CD (Fig. 16A). Así mismo, las crías del grupo HFD mostraron mayor adiposidad en los depósitos de tejido adiposo visceral (Vis) (Fig. 16B) y del subcutáneo (Sc) (Fig. 16C), comparadas con las crías de madres alimentadas con una dieta control.

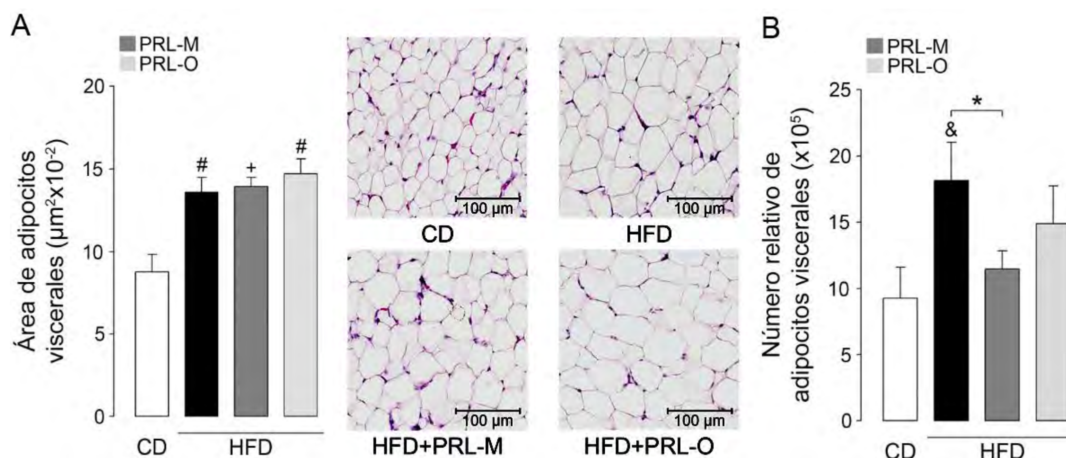

Fig. 17 El consumo materno de una HFD durante la lactancia genera mayor hipertrofia e hiperplasia del tejido adiposo visceral. El tratamiento con PRL en las madres reduce el número de adipocitos viscerales. Área de adipocitos viscerales (A), Número relativos de adipocitos viscerales (B) (n=7-20). Las evaluaciones se realizaron al día 21 de la lactancia en las crías de madres alimentadas con una CD o una HFD, y que recibieron o no el tratamiento materno con PRL (PRL-M) mediante mini-bombas osmóticas subcutáneas, o el tratamiento con PRL en las crías (PRL-O) vía oral durante la lactancia. Se evaluaron en 9 campos por tejido adiposo de 7-20 animales por grupo, y las imágenes presentadas son las representativas de secciones de tejido adiposo teñidas por H&E para evaluar el número y tamaño de los adipocitos. Los datos están representados

por los promedios ± EE. *P<0.05, +P<0.001 vs CD, #P<0.01 vs CD, &P<0.05 vs CD. Las diferencias estadísticas significativas se determinaron con un ANOVA de una vía seguido de una prueba post hoc de Tukey.

Se observó que la adiposidad fue depósito-específica, dado que, en el caso del Vis, la expansión se caracterizó por un incremento en el tamaño (hipertrofia) y número (hiperplasia) de los adipocitos, tal y como puede observarse en la imagen representativa del tejido adiposo Vis de las crías (Fig. 17 A, B). Mientras que, para el Sc, la expansión fue debida a mayor hipertrofia de los adipocitos (Fig. 18A).

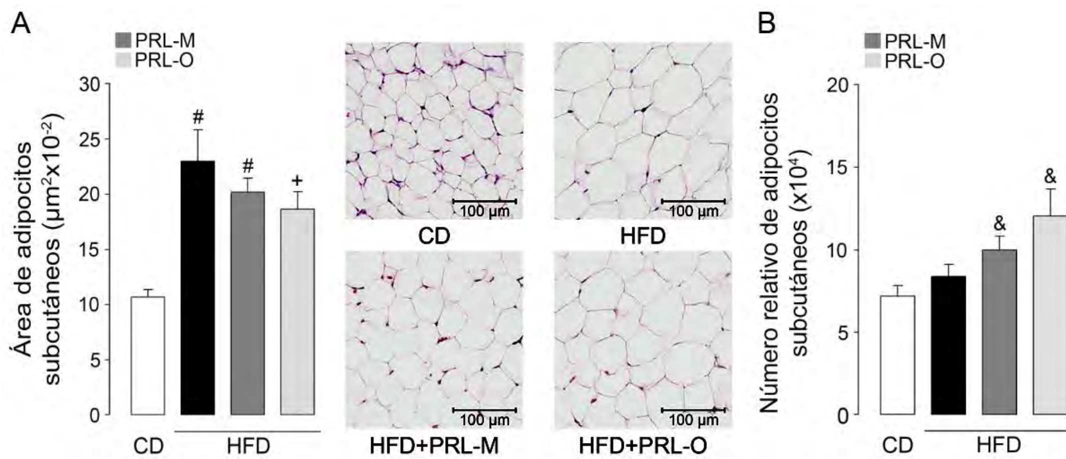

Fig. 18 El consumo materno de una HFD durante la lactancia genera mayor hipertrofia del tejido adiposo subcutáneo. El tratamiento con PRL en las madres o por vía oral en las crías incrementa el número de adipocitos subcutáneos en comparación con las crías en condiciones control. Área de adipocitos subcutáneos (A), Número relativos de adipocitos subcutáneos (B) (n=7-20). Las evaluaciones se realizaron al día 21 de la lactancia en las crías de madres alimentadas con una CD o una HFD, y que recibieron o no el tratamiento materno de PRL (PRL-M) mediante mini-bombas osmóticas subcutáneas, o el tratamiento de PRL en las crías (PRL-O) vía oral durante la lactancia. Se evaluaron en 9 campos por tejido adiposo de 7-20 animales por grupo, y las imágenes presentadas son las representativas de secciones de tejido adiposo teñidas por H&E para evaluar el número y tamaño de los adipocitos. Los datos están representados por los promedios ± EE. +P<0.001 vs CD, #P<0.01 vs CD, &P<0.05 vs CD. Las diferencias estadísticas significativas se determinaron con un ANOVA de una vía seguido de una prueba post hoc de Tukey.

Para determinar sí los efectos benéficos de la PRL sobre la ganancia de peso corporal y la adiposidad, fueron debido a las acciones protectoras de la PRL sobre la funcionalidad de la GM de madres sometidas al reto de una HFD, o debido a la transferencia de la PRL hacia la leche y por tanto a sus acciones directas sobre

las crías, se trató a un grupo de crías de ratas alimentadas con una HFD durante la lactancia con PRL mediante vía oral.

Se encontró que las crías de madres en una HFD y tratadas con PRL (PRL-M), presentaron una disminución significativa en la ganancia de peso corporal (Fig. 16A), y además menor adiposidad Vis (Fig. 16B) representada por una disminución significativa del número de adipocitos viscerales (Fig. 17B) comparadas con las crías de ratas que recibieron una HFD durante la lactancia. Las crías tratadas con PRL oral (PRL-O) presentaron una ganancia de peso corporal similar a las crías provenientes de madres alimentadas con una HFD (Fig. 16A), sin embargo, mostraron una disminución significativa en la adiposidad visceral (Fig. 16B).

Interesantemente no se observaron cambios en la hiperplasia de los adipocitos Sc de las crías de ambos grupos de tratamiento con PRL, PRL-O y PRL-M comparadas con las crías del grupo HFD; sin embargo, en comparación con las crías de ratas alimentadas con CD, el tratamiento con PRL resultó en un incremento significativo de la hiperplasia de los adipocitos del Sc (Fig. 18B).

Estos resultados sugieren fuertemente que la PRL de la leche actúa directamente sobre las crías reduciendo la adiposidad visceral derivada de la alimentación materna obesogénica.

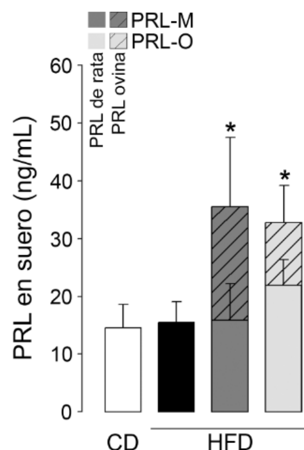

Fig. 19 El tratamiento con PRL en madres (PRL-M) y vía oral en las crías (PRL-O), resulta en un incremento significativo de los niveles de PRL en suero. Las evaluaciones se realizaron al día 21 de la lactancia en las crías de madres alimentadas con una CD o una HFD, y que recibieron o no el tratamiento materno con PRL (PRL-M) mediante mini-bombas osmóticas subcutáneas, o el tratamiento con PRL en las crías (PRL-O) vía oral durante la lactancia. Las barras sin líneas son las correspondientes a la

PRL de rata cuantificadas por radioinmunoensayo, y las barras con líneas son las correspondientes a la PRL ovina cuantificada mediante el ensayo Nb2 (n=8-11). Los datos están representados por los promedios ± EE. *P<0.05. Las diferencias estadísticas significativas se determinaron con un ANOVA de una vía seguido de una prueba post hoc de Tukey.

Como parte de la caracterización de los tratamientos con PRL en las crías (PRL-M y PRL-O), se cuantificaron los niveles de PRL de rata (endógena) y los de PRL ovina (exógena) en las crías (Fig. 19). Se encontró que el tratamiento con PRL oral en las crías resultó en un incremento significativo de los niveles de la hormona en el suero de las crías, similar a los niveles observados en el suero de las crías provenientes de las madres tratadas con PRL.

7.2.2 Niveles de triglicéridos en el hígado de las crías

Otra de las complicaciones derivadas del consumo materno de una dieta alta en grasas a largo plazo es la acumulación de triglicéridos (TAG) en el hígado de las crías lo cual puede promover la formación de hígado graso (Guo et al. 1995). Por lo cual se cuantificaron los TAG hepáticos de las crías, y se encontró que las ratas alimentadas con una HFD durante la lactancia promueven la acumulación de estos componentes de naturaleza lipídica en el hígado de las crías, comparada con los niveles de TAG que mostraron las crías de ratas en CD (Fig. 20A), este resultado se puede observar en las imágenes de las secciones hepáticas teñidas con H&E (Fig. 20B), en donde claramente se observa mayor acumulación de depósitos grasos en comparación con el hígado de crías de madres en CD.

En relación al tratamiento con PRL en las ratas alimentadas con una dieta alta en grasa (HFD), se observó un efecto protector contra la acumulación de triglicéridos en el hígado de sus crías, dado que mostraron una normalización de los niveles de triglicéridos hepáticos (Fig. 20A), lo cual se puede observar en las imágenes de los hígados de las crías (PRL-M) (Fig. 20B), en donde se aprecia menor acumulación de gotas lipídicas en comparación con las crías provenientes de madres que recibieron una dieta alta en grasa.

Mientras que el tratamiento con PRL oral (PRL-O) en las crías de ratas lactantes alimentadas con una HFD, no mostró una disminución significativa de los triglicéridos del hígado (Fig. 20A), sin embargo, se observa claramente menos acumulación lipídica en las secciones histológicas del hígado de dichas crías (Fig. 20B).

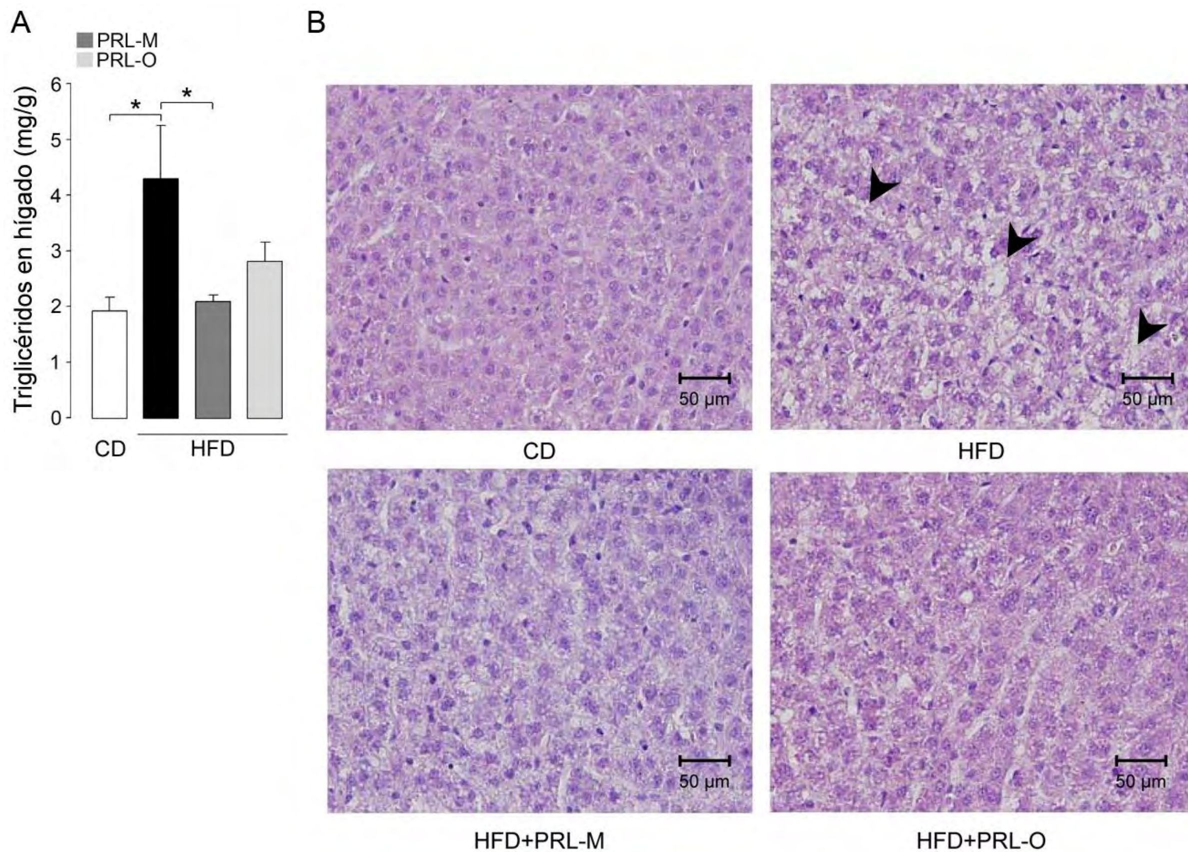

Fig. 20 El tratamiento con PRL previene la acumulación de triglicéridos hepáticos en las crías. Niveles de triglicéridos en hígado (A) (n=10-12), Imágenes representativas de las secciones de hígado teñidas por H&E (B). Las evaluaciones se realizaron al día 21 de la lactancia en las crías de madres alimentadas con una CD o una HFD, y que recibieron o no el tratamiento materno con PRL (PRL-M) mediante mini-bombas osmóticas subcutáneas, o el tratamiento con PRL en las crías (PRL-O) vía oral durante la lactancia. Los datos están representados por los promedios ± EE. *P<0.05. Las diferencias estadísticas significativas se determinaron con un ANOVA de una vía seguido de una prueba post hoc de Tukey.

7.2.3 Evaluación de la señalización de PRL en el hígado

Con el fin de investigar si el tratamiento con PRL en las crías de manera directa (PRL oral) o a través de la madre, ejercía efectos en alguno de los principales tejidos blanco de la hormona, se evaluó la actividad de la PRL en el hígado de las crías, mediante la determinación de la fosforilación de STAT5, el cual es un factor de transcripción parte de la vía de señalización canónica de la PRL (Bole-Feysot et al. 1998). La evaluación se realizó en el hígado, dado que es uno de los tejidos en donde hay mayor expresión de los receptores de PRL (Nagano et al. 1994), y además es uno de los principales tejidos metabólicos en donde se ha reportado la

participación de la prolactina sobre la sensibilidad a la insulina en animales adultos (Yu et al. 2013).

Interesantemente se encontró que las crías provenientes de madres que fueron alimentadas con una HFD durante la lactancia presentaron una disminución en la fosforilación de STAT5 en el hígado, comparada con la observada en las crías bajo condiciones control (Fig. 21A y 21B). Este resultado sugiere que aunque dichas crías mostraron niveles similares de PRL en el suero al día 21 de lactancia, la alimentación alta en grasa, promueve el desarrollo de resistencia a la PRL en el hígado de las crías.

Mientras que ambos tratamientos con PRL, en madres (PRL-M) y en crías (PRL-O), resultaron en una recuperación de la señalización de la prolactina en el hígado, representada por una mayor fosforilación de STAT5 y por lo tanto de su densidad (Fig. 21A, B).

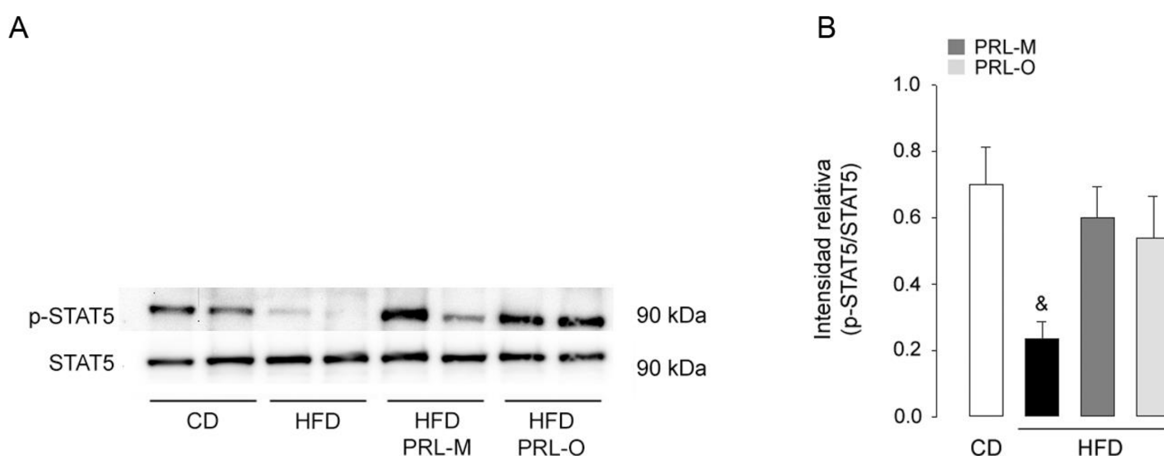

Fig. 21 El tratamiento con PRL previene el desarrollo de resistencia a la prolactina en el hígado de crías provenientes de madres alimentadas con una HFD durante la lactancia. Western Blot de p-STAT5 y STAT5 en hígado (A), Cuantificación de la densidad de las bandas de p-STAT5 y STAT5, (n=8-9) (B). Las evaluaciones se realizaron al día 21 de la lactancia en las crías de madres alimentadas con una CD o una HFD, y que recibieron o no el tratamiento materno con PRL (PRL-M) mediante mini-bombas osmóticas subcutáneas, o el tratamiento con PRL en las crías (PRL-O) vía oral durante la lactancia. Los datos están representados por los promedios ± EE. &P<0.05 HFD vs. todos los grupos. Las diferencias estadísticas significativas se determinaron con un ANOVA de una vía seguido de una prueba post hoc de Tukey.

7.2.4 Sensibilidad a la insulina de las crías lactantes

En ratones, el consumo de dietas altas en grasa durante el periodo de lactancia promueve el desarrollo de hiperinsulinemia en las crías (Vogt et al. 2014). De igual manera, en nuestro modelo, al finalizar la lactancia, las crías de las ratas que recibieron una HFD durante la lactancia, mostraron un incremento del 73% en los niveles de insulina, en comparación con las crías provenientes de madres alimentadas con una CD (Fig. 22A). Así mismo, al evaluar la sensibilidad a la insulina de las crías mediante un ensayo de tolerancia a la insulina (ITT, por sus siglas en inglés), se confirmó la presencia de resistencia a la insulina en las crías del grupo HFD, comparadas con las crías CD, lo cual se puede observar en la mayor área bajo la curva que presentaron dichas crías durante la ITT, lo que señala una disminución significativa en la acción de la insulina para reducir los niveles de glucosa endógena en el animal (Fig. 22B).

Por otro lado, las crías provenientes de madres alimentadas con una HFD pero tratadas con PRL (PRL-M), tuvieron niveles más bajos de insulina al destete, los cuales inclusive llegaron a ser muy similares a los encontrados en las crías de madres alimentadas con una CD (Fig. 22A), y además mostraron una recuperación en su sensibilidad a la insulina durante la ITT (Fig. 22B). En el caso de las crías de madres en HFD y tratadas directamente con PRL oral (PRL-O), aunque presentaron hiperinsulinemia, tuvieron una mejor sensibilidad a la insulina en comparación con las crías de madres en HFD.

Fig. 22 El consumo materno de una HFD promueve el desarrollo de hiperinsulinemia y resistencia a la insulina. Mientras que el tratamiento con PRL en las madres (PRL-M) o en las crías (PRL-O), proporciona protección contra el desarrollo de dichas alteraciones metabólicas. Niveles de insulina en suero de las crías (n= 10-21) (A), ensayo de tolerancia a la insulina (ITT) y el área bajo la curva de la ITT (n=11-22) (B). Las

evaluaciones se realizaron al día 21 de la lactancia en las crías de madres alimentadas con una CD o una HFD, y que recibieron o no el tratamiento materno con PRL (PRL-M) mediante mini-bombas osmóticas subcutáneas, o el tratamiento con PRL en las crías (PRL-O) vía oral durante la lactancia. Los datos están representados por los promedios ± EE. * $P<0.05$, $+P<0.001$ vs. CD, # $P<0.01$ vs. CD, &$P<0.05$ vs. CD y § vs. todos los grupos. Las diferencias estadísticas significativas se determinaron con un ANOVA de una vía o un ANOVA de medidas repetidas (B), seguido de una prueba post hoc de Tukey.

7.2.5 Efectos de la PRL sobre la permeabilidad intestinal de las crías

Recientemente uno de los mecanismos que ha generado atención e interés en el campo de la resistencia a la insulina, es el papel de la permeabilidad intestinal sobre el desarrollo de esta complicación. Se ha reportado que el consumo de dietas altas en grasa altera la microbiota intestinal, lo que activa una serie de respuestas inflamatorias, que en conjunto con la activación del inflamasoma (NLRP3) (Gagliani et al. 2014), promueve la liberación de citocinas proinflamatorias (IL-1β, IL-6, IL-18, entre otras). Esto a su vez debilita la barrera intestinal, debido a que se disminuye la expresión de proteínas de las uniones estrechas de las células epiteliales, como la ZO-1 (zona occludens 1) y la ocludina, lo que da lugar a la transferencia de productos bacterianos como las LPS (lipopolisacáridos de membrana) hacia la circulación, los cuales generan el desarrollo de endotoxemia e inflamación crónica, favoreciendo la resistencia a la insulina y otras complicaciones (Winer et al. 2016).

Por otra parte se conoce que una de las múltiples funciones de la PRL es la regulación del transporte de Ca^{2+} en el intestino, a través de mecanismos transcelulares (Charoenphandhu et al. 2007); más aún se ha encontrado que durante etapas como la gestación y la lactancia, en donde se sabe que la PRL de la madre pasa a la leche materna, y la cual a su vez es capaz de llegar hasta la cría lactante, la PRL puede ejercer acciones sobre la regulación del transporte de Ca^{2+} en las crías neonatales (Amnattanakul et al. 2005). Por todo lo anterior, se evaluaron los efectos de los tratamientos con PRL en las madres (PRL-M) y en las crías (PRL-O), sobre la permeabilidad intestinal de las crías en los días 12 y 21 de la lactancia. Este análisis se hizo a través de la administración oral de la molécula FITC-dextran (Fluorescein isothiocyanate–dextran), la cual por sus propiedades fluorescentes permite la estimación de la permeabilidad intestinal mediante la cuantificación de la cantidad de FITC-dextran que pasa del intestino al suero sanguíneo (Moussaoui et al. 2014).

Interesantemente se encontró que las crías provenientes de madres que recibieron una HFD durante la lactancia presentaron un incremento significativo en

la permeabilidad intestinal al día 12 de la lactancia, comparadas con las crías de ratas alimentadas con una CD (Fig. 23A). Mientras que al día 21 de la lactancia no se observaron cambios en la permeabilidad intestinal de las crías (Fig. 23B).

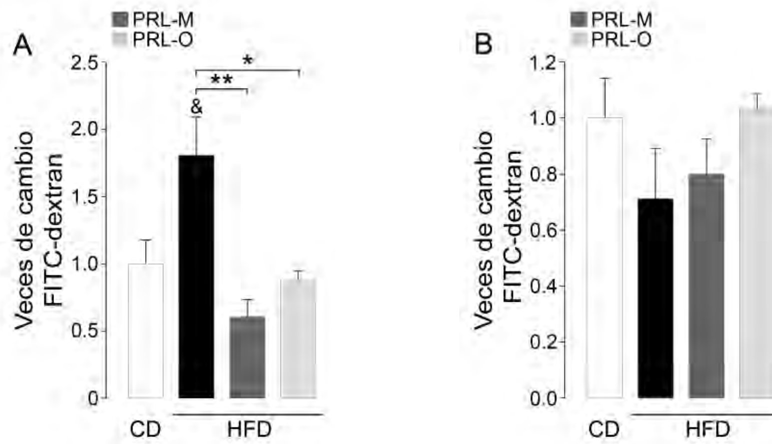

Fig. 23 El consumo materno de una HFD durante la lactancia, incrementa la permeabilidad intestinal de las crías, mientras que el tratamiento con PRL en las madres (PRL-M) o en las crías (PRL-O), previene alteraciones en la permeabilidad intestinal en la fase temprana de la lactancia. Estimación relativa de los niveles de FITC-dextran en suero de las crías al día 12 (A) y 21 (B) de la lactancia (n=7-8). Las evaluaciones se realizaron en las crías de madres alimentadas con una CD o una HFD, y que recibieron o no el tratamiento materno con PRL (PRL-M) mediante mini-bombas osmóticas subcutáneas, o el tratamiento con PRL en las crías (PRL-O) vía oral durante la lactancia. Los datos están representados por los promedios ± EE. * $P<0.05$, ** $P<0.01$, & $P<0.05$ vs. CD. Las diferencias estadísticas significativas se determinaron con un ANOVA de una vía, seguido de una prueba post hoc de Tukey.

Por su parte, las crías provenientes de madres alimentadas con una HFD que fueron tratadas con la PRL (PRL-M), o a las que se les proporcionó PRL por vía oral (PRL-O), presentaron una disminución significativa de la permeabilidad intestinal, en comparación con las crías del grupo HFD (Fig. 23A) al día 12 de lactancia. Mientras que al día 21, no se encontraron diferencias significativas en los grupos (Fig. 23B). Estos datos muestran que la PRL ejerce una función de protección contra las alteraciones en la permeabilidad intestinal derivadas del consumo de leche materna proveniente de ratas alimentadas con una HFD. Así mismo este resultado podría estar asociado con la mejor sensibilidad a la insulina (Fig. 22B) que presentaron las crías sometidas a los tratamientos con PRL a través de sus madres o de manera directa.

7.2.6 Marcadores intestinales de la respuesta inflamatoria inducida por el consumo de una dieta alta en grasas

Debido a que el consumo de dietas altas en grasas promueven un incremento en la respuesta inflamatoria en el intestino, lo que da lugar al desarrollo de complicaciones como resistencia a la insulina, se determinó la expresión de algunos genes de citocinas proinflamatorias en colon y yeyuno (*Il1β, Tnfα y Tgfβ1*), así como del glucagón (*Gcg*) que en respuesta a la absorción de nutrientes da lugar a la formación del GLP-1 (Glucagon like peptide 1), el cual estimula la secreción de insulina (Uccellatore et al. 2015).

Los resultados obtenidos muestran que las crías provenientes de madres a las que se les alimentó con una HFD durante la lactancia, presentaron mayor inflamación intestinal en comparación con las crías de ratas lactantes que recibieron una dieta control, dado que presentaron mayor expresión de la citocina proinflamatoria TNF-α (*Tnf*) en yeyuno (Fig. 24A), así como de la citocina IL1β (*Il1b*), mientras que se encontró mayor expresión del mediador antiinflamatorio TGF-β1 (*Tgfb1*), lo que muestra la presencia del proceso de inflamación crónica derivado de la dieta de sus madres (Fig. 24B). Mientras que en el caso del glucagón se observó una disminución significativa en la expresión de este gen en el colon de las crías del grupo HFD en comparación con las crías provenientes de madres en CD (Fig. 24B).

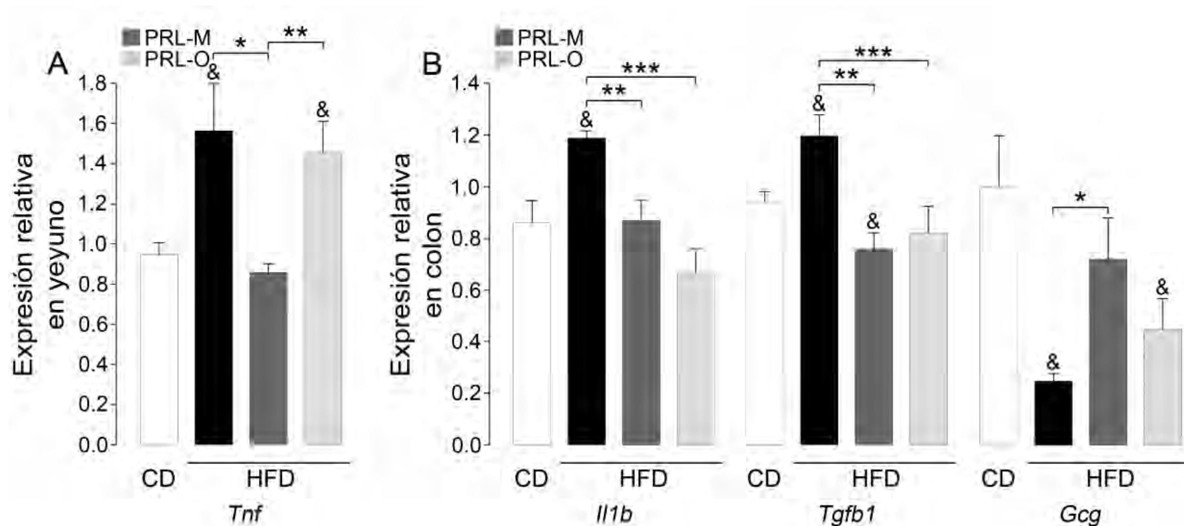

Fig. 24 El consumo materno de una HFD durante la lactancia, promueve la respuesta inflamatoria en el intestino de sus crías. El tratamiento con PRL en las madres alimentadas con una HFD durante la lactancia, confiere protección antiinflamatoria en el intestino de sus crías. Expresión relativa de la citocina

proinflamatoria TNF-α (*Tnf*) en el yeyuno de las crías (A) y expresión relativa de la citocina proinflamatoria IL1β (*Il1b*), del factor de crecimiento TGF-β1 (*Tgfb*) y del glucagón (*Gcg*) en el colon de las crías (B) de la lactancia (n=6-10). Las evaluaciones se realizaron al día 12 de la lactancia en las crías de madres alimentadas con una CD o una HFD, y que recibieron o no el tratamiento materno con PRL (PRL-M) mediante mini-bombas osmóticas subcutáneas, o el tratamiento con PRL en las crías (PRL-O) vía oral durante la lactancia. Los datos están representados por los promedios ± EE. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, & $P<0.05$ vs. CD. Las diferencias estadísticas significativas se determinaron con un ANOVA de una vía, seguido de una prueba post hoc de Tukey.

En concordancia con el resultado observado en la permeabilidad intestinal, las crías provenientes de madres alimentadas con una HFD tratadas con PRL mediante el implante de mini-bombas osmóticas, presentaron una disminución significativa de la expresión de citocinas proinflamatorias tanto en el yeyuno como en el colon, así como menor expresión de la respuesta antiinflamatoria representada por TGF-β1 y mayor expresión del glucagón, en comparación con las crías de ratas lactantes que recibieron una HFD (Fig. 24A, B). Mientras que las crías tratadas por vía oral con la PRL presentaron menor expresión de la IL1β en colon, así como de TGF-β1, lo que sugiere que el mecanismo de acción de la PRL sobre la protección en la permeabilidad intestinal tiene una respuesta sinérgica con los otros componentes de la leche materna, tal y como lo muestran los resultados obtenidos en las crías de madres tratadas con PRL (PRL-M).

8. DISCUSIÓN

En las últimas 3 décadas, la prevalencia de obesidad se ha incrementado de manera exponencial a nivel mundial en todos los rangos de población, desde niños hasta adultos, en donde además se ha observado mayor prevalencia de sobrepeso y obesidad en mujeres que en hombres; siendo de gran importancia en mujeres durante la edad reproductiva por la implicación que posee la patología y sus complicaciones sobre la descendencia (Perez Ferrer et al. 2014, Hernández-Avila M 2016).

Al respecto, se han estudiado ampliamente los efectos de la obesidad derivada de una mal nutrición materna (entendiéndose como el consumo de dietas hipercalóricas, provenientes de carbohidratos y grasas principalmente) durante etapas como la gestación y lactancia, sobre el metabolismo de su progenie. Se ha encontrado que el consumo materno de dietas altas en grasa durante el embarazo y lactancia (principal componente de la dieta hipercalórica utilizada en este proyecto), promueve el desarrollo de alteraciones metabólicas en sus crías, caracterizadas por mayor peso corporal, mayor adiposidad, hiperinsulinemia, hígado graso y resistencia a la insulina (Du et al. 2012, Sun et al. 2012, Masuyama et al. 2014, Vogt et al. 2014, Bautista et al. 2016). Así mismo, este tipo de dietas también producen alteraciones en la morfología y función de la glándula mamaria, lo que además se ve reflejado en cambios tanto en la composición de la leche como en su producción (Du et al. 2012, Hernandez et al. 2012, Bautista et al. 2016).

Sin embargo, se ha estudiado poco la identidad y contribución de los componentes de la leche materna que se alteran ante un reto nutricional en la madre durante la etapa de la lactancia, sobre la programación metabólica en sus crías. Por lo cual en este proyecto se planteó evaluar el papel metabólico de una de las principales hormonas reguladoras de la lactancia, la PRL, en un ambiente obesogénico proveniente de la leche materna. Encontramos que los efectos metabólicos adversos sobre las crías derivados de la ingesta de una dieta alta en grasas (HFD) por parte de sus madres, involucran daños en la función de la PRL sobre el mantenimiento de la estructura y funcionalidad de la glándula mamaria, lo que causa alteraciones en la producción y composición de la leche, y, además, reduce los niveles de PRL de la leche, dando lugar a que los efectos metabólicos benéficos de la PRL sobre las crías se vean reducidos.

En humanos y en roedores se ha encontrado que la obesidad reduce la eficiencia de la lactancia, que involucra un retardo en el inicio de la secreción de leche, la involución temprana del tejido mamario, y una disminución en la producción de

leche (Rasmussen et al. 2004, Turcksin et al. 2014, O'Sullivan et al. 2015, Buonfiglio et al. 2016). En concordancia, en modelos animales, el consumo de una HFD desde antes del apareamiento y durante el embarazo y la lactancia, resulta en alteraciones en la morfología y función de glándula mamaria, así como en la eficiencia de la lactancia (Flint et al. 2005, Hernandez et al. 2012, Buonfiglio et al. 2016).

Los mecanismos involucrados en las alteraciones generadas por el consumo de una HFD en el embarazo y la lactancia, incluyen una disminución en las acciones de la PRL en respuesta a su principal estímulo, la succión de las crías (Rasmussen et al. 2004), incremento en la producción de serotonina y de procesos inflamatorios en la glándula mamaria, dando lugar al proceso de involución (Stull et al. 2007, Hernandez et al. 2012) y al desarrollo de resistencia a la PRL, tanto en el hipotálamo como en la propia glándula mamaria, lo cual se demostró por un incremento en los niveles de la proteína fosforilada de STAT5 en el tejido mamario de ratonas lactantes con obesidad, y la falta de una mayor fosforilación de STAT5 ante una inyección de PRL (Buonfiglio et al. 2016).

Con el diseño experimental de este proyecto, en el cual se proporcionó una HFD solo durante la lactancia, se pudieron evaluar de manera selectiva los efectos del consumo excesivo de grasas durante este periodo, sin la influencia de alteraciones derivadas de la obesidad durante la gestación. Y en relación a esto, se encontró que aun cuando el desarrollo de la glándula mamaria durante la gestación ocurrió en condiciones normales, el consumo de una HFD durante la lactancia generó alteraciones similares a las encontradas en modelos de obesidad a largo plazo, como disminución del área parenquimal y mayor área adiposa, asociándose con un incrementó en la inflamación de la glándula mamaria y marcadores de involución (Flint et al. 2005, Hernandez et al. 2012), estos resultados muestran que la etapa de la lactancia, es el periodo más sensible para el desarrollo de alteraciones en la funcionalidad de la glándula mamaria.

Encontramos que las alteraciones en la estructura y función de la glándula mamaria derivados del consumo de una dieta alta en grasa, son generados por una disminución de la función de la PRL en el tejido mamario. Esta situación puede ser debido al desarrollo de resistencia a la PRL (Buonfiglio et al. 2016), sin embargo también podría ser causa de una disminución en la sensibilidad a la PRL en la glándula mamaria, dado que la HFD produjo una reducción significativa en la expresión del receptor de PRL en dicho tejido; más aún, ésta disminución en la expresión del receptor de PRL podría estar asociada con el desarrollo de resistencia a la PRL; sin embargo a diferencia de otras hormonas, en donde al existir resistencia, los niveles de las hormonas se encuentran elevados (insulina y

leptina) (Munzberg 2010, Czech 2017), en este caso no se encontraron niveles elevados de la PRL en la circulación.

Debido a que las alteraciones en la GM inducidas por el consumo de una HFD son prevenidas por el tratamiento con PRL, se fortalece la teoría de que daños en la eficiencia de la lactancia debidos a la obesidad o a una ingesta excesiva de alimento, involucra una disminución de las acciones de la PRL, lo cual sugiere el uso de un tratamiento con PRL en mujeres con obesidad y problemas para lactar.

Los mecanismos propuestos para la protección ejercida por la PRL contra las alteraciones en la morfología y función de la glándula mamaria derivadas del consumo de una HFD durante la lactancia, son los correspondientes a los efectos canónicos de la hormona, como la promoción y mantenimiento de la diferenciación de la glándula mamaria (Brisken et al. 1999, Hennighausen et al. 2001, Gillam et al. 2011), y el retardo en el proceso de involución inducida por el consumo de una HFD (Flint et al. 2005, Du et al. 2012, Hernandez et al. 2012). Dado que el consumo materno de una HFD durante la lactancia genera una reducción en los niveles de PRL de la leche, mientras que el tratamiento con PRL en condiciones de una HFD resultó en la normalización de los niveles de PRL en la leche, muestra que la PRL previene las alteraciones en la glándula mamaria derivadas de la HFD, lo que facilita la transferencia de PRL del suero a la leche, y/o la propia síntesis de la hormona en la glándula mamaria, lo que a su vez permite el adecuado mantenimiento de la lactancia.

Así mismo, la obesidad o la ingesta excesiva de nutrientes generan cambios en la composición de la leche, dentro de los cuales, los más estudiados han sido los ácidos grasos, hormonas, citocinas, entre otros. De hecho se ha reportado que la leche de madres con obesidad, tanto en humanos como en modelos murinos, tiene un alto contenido de ácidos grasos saturados de cadena larga y además niveles elevados de insulina, leptina y citocinas proinflamatorias (Rolls et al. 1986, Oben et al. 2010, Du et al. 2012, Du et al. 2015, Bautista et al. 2016, De Luca et al. 2016, Lemas et al. 2016, Young et al. 2017).

Es conocido que una de las funciones principales de la PRL durante la lactancia es regular la composición de la leche, dado que promueve la expresión de varias proteínas de la leche, como la β-caseína (Guyette et al. 1979), la proteína ácida de la leche (Hennighausen et al. 1991), la α-lactoalbúmina (Jagoda et al. 1991) y la lactoglobulina (Collet et al. 1991); además promueve la síntesis de lactosa y por lo tanto aumenta la producción de leche (Oppat et al. 1988, Powe et al. 2010). Adicionalmente, incrementa los niveles de calcio (Villalba et al. 1991, Powe et al. 2011) y reduce los de sodio (Powe et al. 2011), dando lugar a la reducción en la

permeabilidad del epitelio de la glándula mamaria (Nguyen et al. 1998, Powe et al. 2011).

Además, se conoce que la PRL regula la expresión de enzimas involucradas en la síntesis de ácidos grasos, como la acetil-CoA carboxilasa, la ácido graso sintasa, la acetil-CoA sintetasa y la piruvato deshidrogenasa (Waters et al. 1988, Rudolph et al. 2011, Ben-Jonathan et al. 2015); por lo tanto está directamente implicada con los niveles de ácidos grasos en el tejido mamario, los cuales pueden ser depositados directamente en la leche. Algunos ácidos grasos presentes en la leche materna, poseen propiedades funcionales (Diau et al. 2005, Heird et al. 2005, Ganapathy 2009), dentro de los más estudiados se encuentran los ácidos grasos poliinsaturados, como el ácido docosahexaenoico o DHA por sus siglas en inglés, el ácido araquidónico, y recientemente han sido objeto de estudio por sus notables propiedades metabólicas los ésteres de ácidos grasos ramificados en su radical hidroxilo (FAHFAs, Branched Fatty Acid Esters of Hydroxy Fatty Acids) (Yore et al. 2014).

Además, se conoce que la PRL incrementa los niveles de oligosacáridos de la leche, los cuales son glicanos resistentes a la digestión gastrointestinal, lo que les permite llegar hasta el colon, en donde actúan como prebióticos. Así mismo, estos compuestos inhiben la adhesión de bacterias patógenas y sus toxinas en el tracto gastrointestinal y contribuyen con el desarrollo intestinal del infante (Powe et al. 2011, Musilova et al. 2014, Andreas et al. 2015). Todo esto apoye que a través de la regulación de la composición de la leche y la funcionalidad de la glándula mamaria, la PRL es capaz de influenciar el metabolismo del infante.

Uno de los objetivos principales del proyecto fue investigar si el consumo de una dieta alta en grasas durante la lactancia podría alterar los niveles de PRL en la leche. Los resultados muestran que el exceso de grasa en la alimentación de las madres lactantes, resultó en una reducción de los niveles de PRL en la leche; mientras que los niveles de la hormona en el suero no fueron alterados por la dieta, lo cual sugiere que la reducción de PRL en la leche es debida a una menor transferencia de la circulación a la leche. Dicha transferencia ocurre por endocitosis mediada por el receptor de PRL (Ollivier-Bousquet et al. 1993, Seddiki et al. 2002), e interesantemente se encontró que así como disminuyeron los niveles de PRL en la leche, la expresión del receptor de PRL en la glándula mamaria también se redujo en el grupo de ratas lactantes que recibieron una HFD. Mientras que dichos valores fueron normalizados por el tratamiento con PRL, mostrando que el consumo de una HFD durante la lactancia resulta en una reducción de la expresión del receptor de la hormona en la glándula mamaria, lo que contribuye con la disminución de los niveles de PRL en la leche.

Otro de los objetivos centrales del proyecto era dilucidar los efectos metabólicos de la PRL sobre las crías, dado que se ha reportado que la PRL es transportada hacia la circulación de las crías alimentadas con leche materna (Whitworth et al. 1978, Gonnella et al. 1989). En relación a esta transferencia, es conocido que ocurre en las porciones del yeyuno y del íleon mediante un mecanismo transepitelial (Gonnella et al. 1989). Así mismo trabajos en donde a madres lactantes se les administró PRL marcada radioactivamente, se encontró a la PRL en el suero de sus crías después de la succión de leche (Whitworth et al. 1978). Aunque es interesante destacar que los niveles de PRL reportados en el suero de las crías lactantes son bastante bajos en las primeras dos semanas de vida (Shah et al. 1988), sugiriendo que las principales acciones metabólicas de la hormona proveniente de la leche podrían ocurrir a nivel gastrointestinal.

En relación a los efectos conocidos de la PRL de la leche sobre crías lactantes, se ha propuesto que regula el desarrollo de sistemas como el neuroendocrino, inmune y reproductivo (Grove et al. 1991, Ellis et al. 1996, Melo et al. 2009) tal y como se muestra en algunos experimentos en donde se utilizó bromocriptina como agonista dopaminérgico para reducir los niveles de PRL en el suero y por lo tanto en la leche de madres lactantes. Sin embargo, con los datos mostrados en esos trabajos no es posible determinar si los efectos observados en las crías son debidos a la reducción de PRL o a la bromocriptina transferida hacia la leche (Bonomo et al. 2007, de Moura et al. 2009). A diferencia de trabajos previos, en este proyecto, la PRL se administró directamente a la madre lactante o a las crías, lo cual permitió analizar de manera específica los efectos de la hormona sobre las crías, mostrando que la PRL de la leche regula positivamente el metabolismo de las crías lactantes.

Con relación a efectos de otras hormonas de la leche sobre las crías, se ha reportado que hormonas como la leptina, FGF21, insulina, entre otras, han mostrado efectos directos sobre las crías lactantes (Sanchez et al. 2005, Vogt et al. 2014, Gavalda-Navarro et al. 2015). Específicamente se ha encontrado que la administración de leptina en crías lactantes reduce la ingesta de alimento y disminuye la ganancia de peso y adiposidad derivada de una HFD en la etapa adulta (Pico et al. 2007). A diferencia de la mayoría de las hormonas, la PRL se encuentra en mayor concentración en la leche que en la circulación materna (Aydin et al. 2008, Bonomo et al. 2008, Gavalda-Navarro et al. 2015, Badillo-Suarez et al. 2017). Mas aún la PRL presente en la leche es transferida hacia la circulación de las crías lactantes (Whitworth et al. 1978), dicha PRL podría ser capaz de ejercer acciones directas en los principales tejidos metabólicos de las crías, como el tejido adiposo, hígado y páncreas (Park et al. 2011, Yu et al. 2013, Ruiz-Herrera et al. 2017). Aunque no se ha descrito completamente la presencia

del receptor de PRL en dichos tejidos durante etapas tempranas, recientemente se reportó que en las primeras 2 semanas postnatales, el hígado produce PRL e incrementa la expresión de su receptor, sugiriendo el papel regulador de la PRL sobre el desarrollo del hígado en la etapa postnatal (Moreno-Carranza et al. 2018).

Los efectos del tratamiento con PRL demuestran que la hormona participa en el metabolismo del tejido adiposo de las crías. En particular se encontró que las crías tratadas oralmente con PRL y provenientes de madres en HFD presentaron menor adiposidad visceral y mayor hiperplasia del tejido adiposo subcutáneo, en concordancia con este resultado, en un trabajo previo, se encontró que en ratas macho adultas con obesidad, el tratamiento de PRL redujo la hipertrofia del tejido adiposo visceral, e incrementó la hiperplasia de los adipocitos (Ruiz-Herrera et al. 2017).

Además, las crías provenientes de madres tratadas con PRL presentaron menor acumulación de triglicéridos en el hígado en comparación con las crías de madres en HFD, se sugiere que la PRL tiene efectos específicos sobre dicho tejido. En relación a esto, se ha encontrado en animales adultos, que la PRL estimula la sensibilidad a la insulina en el hígado, efecto que ocurre mediante la activación de STAT5, que forma parte de la vía de señalización canónica de la PRL (Yu et al. 2013); los mecanismos implicados en la regulación de la sensibilidad a la insulina se sugiere que podrían ser varios, el primero sería a través de STAT5, modulador a su vez de la activación de STAT1 y STAT3, los cuales incrementan la expresión de IGF-1 y activan a la quinasa PI3K; por otro lado también se ha encontrado que el receptor de PRL interacciona con IRS-1 y PI3K, activando además fosforilaciones en residuos de tirosina (Berlanga et al. 1997, Santos et al. 2001, Cui et al. 2007).

Acorde con los efectos de la PRL reportados en el hígado, se encontró que los tratamientos con PRL en las madres o de manera oral en las crías, incrementaron la fosforilación de STAT5 en el hígado de las crías, en comparación con lo observado en las crías de madres en HFD, que presentaron una reducción significativa en la fosforilación de la proteína, comparadas con las crías de madres en CD. Esto refleja que parte de los efectos metabólicos de la PRL de la leche son favorecer la sensibilidad a la insulina de las crías, mediante la activación de la cascada de señalización de la PRL en el hígado, la cual promueve la sensibilidad a la insulina (Yu et al. 2013).

Si bien los efectos de la PRL en las crías favoreciendo la sensibilidad a la insulina, podrían explicarse por acciones directas de la PRL proveniente de la leche sobre los principales tejidos metabólicos, tal y como se observó en la disminución

significativa de la adiposidad visceral, y a través de disminuir la acumulación de triglicéridos en el hígado; estos efectos también podrían verse extendidos a otros tejidos involucrados en la homeostasis metabólica, como el músculo, páncreas, hipotálamo, intestino, entre otros (Haeusler et al. 2018).

En los últimos años se ha estudiado la regulación de la permeabilidad intestinal y sus implicaciones sobre el desarrollo de resistencia a la insulina derivada del consumo de dietas altas en grasa. Específicamente se ha encontrado que este tipo de alimentación genera alteraciones en la población bacteriana de la microbiota intestinal, lo que promueve procesos inflamatorios, los cuales reducen la expresión de proteínas de membrana en el epitelio del intestino, dando lugar a que se incremente la permeabilidad intestinal, esto permite la entrada de LPS y otros restos bacterianos, así como citocinas proinflamatorias a la circulación, promoviendo un estado de inflamación crónica que contribuye con la resistencia a la insulina (Winer et al. 2016). Mas aún se ha reportado que durante la gestación y lactancia, la PRL regula el transporte intestinal de Ca^{2+} en las crías neonatales (Amnattanakul et al. 2005), lo que sugiere que la PRL tiene efectos sobre la permeabilidad intestinal. Nuestros resultados muestran que la PRL de la leche favorece el mantenimiento de la permeabilidad intestinal ante un reto hipercalórico proveniente de una alimentación alta en grasas en las madres lactantes. Estas acciones de la PRL en el intestino podrían contribuir con los efectos benéficos de la hormona favoreciendo la sensibilidad a la insulina, tal como se observó en las crías tratadas con PRL.

El principal objetivo de este proyecto fue investigar los efectos metabólicos de la PRL durante la lactancia sobre las crías expuestas a condiciones obesogénicas, para lo cual se usó como reto nutricional el consumo materno de una dieta alta en grasas durante la lactancia. Para evaluar el efecto de la PRL, se utilizaron dos tratamientos con PRL, el primero con la finalidad de restituir los niveles reducidos de la PRL en la leche inducidos por la ingesta de una HFD, a través de la administración oral en las crías, y el otro con el propósito de compensar las acciones reducidas de la PRL sobre la función de la glándula mamaria y por ende la eficiencia de la lactancia, para lo cual se les implantó a las madres lactantes una mini-bomba osmótica conteniendo a la PRL.

Con los resultados obtenidos en este proyecto de investigación se logró demostrar que la PRL durante la lactancia juega un papel relevante sobre la regulación metabólica de las crías lactantes. Dentro de las acciones metabólicas directas de la PRL ante un reto obesogénico, se encontró que reduce la adiposidad visceral y estimula la sensibilidad a la insulina de las crías.

Otros de los efectos de la PRL se deben a acciones secundarias de la hormona sobre las crías, ya que participa en el mantenimiento de la funcionalidad del tejido mamario y en la regulación de los componentes de la leche, dado que se observaron algunos efectos en las crías provenientes de madres tratadas con PRL que no mostraron las crías a las que se les administró de manera oral la PRL, como la ganancia de peso corporal y los niveles de insulina en la circulación. Adicionalmente, algunos efectos de la PRL en las crías podrían deberse a la combinación de las acciones directas e indirectas de la PRL de la madre, como la acumulación de triglicéridos en el hígado y los niveles de insulina, en donde el efecto de la PRL fue mayor en las crías provenientes de madres tratadas con PRL, mientras que el tratamiento con PRL oral ejerció efectos parciales sobre estos parámetros.

En resumen, durante la lactancia la PRL participa en la regulación del metabolismo de las crías neonatales, mediante efectos indirectos a través de la leche materna y directamente sobre los tejidos metabólicos de las crías. La PRL actúa promoviendo la diferenciación y función de la glándula mamaria, permitiendo que se lleve a cabo la lactancia, así como en la regulación de la composición y producción de la leche. La leche materna contiene altos niveles de PRL, la cual llega a la circulación de las crías y, regula su homeostasis metabólica. Además, los efectos deletéreos del consumo materno de una dieta alta en grasa durante la lactancia sobre el metabolismo de las crías involucran alteraciones en las funciones de la PRL tanto en la madre como directamente en las crías.

9. CONCLUSIÓN

La nutrición materna durante etapas críticas del desarrollo, como la lactancia ejerce un papel fundamental en la determinación del estado fisiológico de la cría lactante. En este proyecto se evaluó la función de una de las principales hormonas reguladoras de la lactancia, la PRL, y se encontraron hallazgos relevantes sobre su función en el establecimiento de la salud metabólica de las crías. Debido a que la leche materna es un alimento con una amplia variedad de componentes que han sido relacionados con propiedades funcionales, resulta de interés su estudio en relación con sus efectos protectores contra el desarrollo de alteraciones en la homeostasis metabólica de las crías.

El consumo materno de una dieta alta en grasas durante la lactancia altera la funcionalidad de la PRL sobre el tejido mamario, reduciendo la expresión del receptor de PRL, lo que contribuye con los efectos adversos de la dieta obesogénica sobre el metabolismo de las crías.

Por otro lado, la PRL de la leche materna participa de manera directa (leche materna) e indirecta (funcionalidad de la glándula mamaria) sobre la regulación del metabolismo de las crías y estos efectos se ven alterados por una dieta materna obesogénica.

Los efectos de la PRL de la leche sobre el metabolismo de las crías involucran efectos directos en la permeabilidad y función intestinal, así como en otros tejidos metabólicos como el hígado y el tejido adiposo, en estos últimos los efectos podrían ser directos o indirectos. En conjunto, estos efectos explican las acciones de la PRL favoreciendo la sensibilidad a la insulina en las crías, incluso ante un reto nutricional hipercalórico derivado de la dieta materna.

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Impaired prolactin actions mediate altered offspring metabolism induced by maternal high-fat feeding during lactation

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ABSTRACT: Maternal diet during lactation affects offspring metabolic health throughout life. Prolactin (PRL) is present in high quantities in maternal milk; however, the effects of milk PRL on the offspring remain poorly characterized. In this study, we evaluated whether feeding a high-fat diet (HFD) to rats during lactation alters PRL, both in the mother's serum and in milk, and whether this factor contributes to HFD-induced metabolic dysfunction in the offspring. Maternal HFD resulted in decreased PRL levels in milk (but not in serum), reduced mammary gland (MG) PRL receptor expression, and altered MG structure and function. Offspring from HFD-fed dams had increased body weight and adiposity, and developed fatty liver, hyperinsulinemia, and insulin resistance at weaning. Increasing PRL levels in the HFD-fed mothers by subcutaneous osmotic minipumps releasing PRL normalized MG function and PRL levels in milk. Moreover, PRL treatment in HFD-fed mothers, or directly in their pups *via* oral PRL administration, increased liver STAT5 phosphorylation, reduced visceral adiposity, ameliorated fatty liver, and improved insulin sensitivity in offspring. Our results show that HFD impairs PRL actions during lactation to negatively affect MG physiology and directly impair offspring metabolism.—De los Ríos, E. A., Ruiz-Herrera, X., Tinoco-Pantoja, V., López-Barrera, F., Martínez de la Escalera, G., Clapp, C., Macotela, Y. Impaired prolactin actions mediate altered offspring metabolism induced by maternal high-fat feeding during lactation. FASEB J. 32, 000–000 (2018). www.fasebj.org

KEY WORDS: nursing pups · metabolic programming · insulin resistance

Breastfeeding is an early determinant of lifetime health status. Breastfed children have lower mortality rates associated with infectious diseases and a lower prevalence of obesity and type 2 diabetes in both childhood and adulthood (1, 2). However, given the current pandemics of overweight and obesity, a high proportion of women have unhealthy weight and poor nutrition habits during their reproductive lifetime. The diet of the mother affects milk composition and the metabolic status of the offspring (3–7). Even in conditions of normal body weight during gestation, a maternal high-fat diet (HFD) during lactation alters offspring metabolism as revealed by the increased body weight, higher adiposity, glucose intolerance, hyperinsulinemia, and insulin resistance of the nursing young (5, 6, 8). Breast milk contains all the nutrients needed for the adequate growth and development of the newborn, including many functional components, such as immunoglobulins and fatty acids (FA) (arachidonic acid and docosahexaenoic acid) that promote immunity and neurologic development, respectively (9–12). Maternal milk also contains many hormones that regulate metabolism: insulin, leptin, adiponectin, ghrelin, fibroblast growth factor 21 (FGF21), and prolactin (PRL) (13–16). It has been suggested that these hormones mediate many of the beneficial effects of breast milk (13, 14, 16–18).

PRL, one of the main determinants of lactation (19, 20), regulates metabolic homeostasis. PRL levels decrease in the circulation of HFD-fed adult rats and insulin-resistant humans (21–23), and elevating circulating levels of PRL in obese rats improve their insulin sensitivity (23). PRL is present in milk in high quantities (24, 25), and milk PRL can reach the circulation of the nursing young (26, 27); however, the role of milk PRL in the offspring metabolism remains unknown. It has been suggested that milk PRL may regulate the neuroendocrine, reproductive, and immune systems in the lactating neonates (16, 28, 29), but direct evidence is lacking.

ABBREVIATIONS: BWG, body weight gain; CD, control diet; FA, fatty acids; FGF21, fibroblast growth factor 21; HFD, high-fat diet; ITT, insulin tolerance test; MG, mammary gland; PRL, prolactin; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue

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The present study investigated whether an HFD in rats alters PRL levels in maternal serum and milk during lactation and whether such alterations contribute to the harming effects of an HFD on the metabolism of the nursing young. Our results show for the first time, to the best of our knowledge, a dual role for maternal PRL in regulating the offspring metabolism, both indirectly, by acting on the mother to regulate mammary gland (MG) function and milk composition, and directly by acting on the pups *via* the milk. Moreover, we show that PRL actions are impaired by HFD during lactation, with deleterious consequences for the offspring.

MATERIALS AND METHODS

Animals

Female Wistar rats (300–350 g) were housed at 22°C under a 12-h light/dark cycle with free access to food and water. Animals received a control diet (CD) (2.91 kcal/g, in which 28, 13.5, and 58% of calories comes from proteins, fat, and carbohydrates, respectively) (Laboratory Rodent Diet 5001; LabDiet, Richmond, IN, USA) before and during the gestation period. One day after giving birth (defined as d 1 of lactation), litters were adjusted to 8 pups per dam, and were randomly assigned to a CD or an HFD (5.24 kcal/g, in which 20, 60, and 20% of calories comes from proteins, fat, and carbohydrates) (OpenSource Diet D12492; Research Diets, New Brunswick, NJ, USA) for the 21 d of lactation. On d 4 of lactation, animals were anesthetized with inhaled ether (for an optimal surgery and a fast recovery) and sham-treated or implanted subcutaneously with Alzet osmotic minipumps (Durect Corp., Cupertino, CA, USA) releasing 0.16 mg/kg/d of ovine PRL (Sigma-Aldrich, St. Louis, MO, USA) for the rest of the lactation period (d 21). In addition, a subgroup of offspring from mothers of the HFD-fed group received ovine PRL orally once a day (20 μl at a concentration mimicking the levels of PRL present in maternal milk throughout lactation, starting with 1000 ng/ml on lactation d 4, and gradually decreasing 50 ng/d to reach 150 ng/ml on d 21 of lactation).

The rest of the offspring (in all groups) received 20 μl of vehicle orally (PBS) and were subjected to the same manipulation and treatment to avoid confounding factors induced by the handling and by the oral treatment itself. During the procedure, the pups were held softly facing up, and the skin on the back of the neck was held between 2 fingers to open the pup's mouth. A soft cannula (1 mm outer diameter and 0.5 mm inner diameter) connected to a syringe was inserted ~0.5 cm into the oral cavity of the rat, and the liquid was delivered. Aware of the sensitivity to stress of the dams and pups around birth time, the pups and dams were left untreated from postnatal d 1 (after changing the diet and adjusting the litter) to d 3 to allow lactation and bonding to initiate and stabilize and, thereby, prevent dams from abandoning their pups due to stress. Also, to avoid stress-induced PRL release, animals were handled daily for 7 d before euthanization by CO_2 inhalation followed by decapitation. We used a total of 79 dams and examined 253 pups. Two to 4 pups per dam were randomly selected to obtain tissues and serum and to perform insulin tolerance tests (ITTs). To our knowledge, there were no confounding factors when examining or selecting the pups.

All animal procedures were approved by the Bioethics Committee of the Institute of Neurobiology of the National University of Mexico (Protocol 075) and comply with the *Guide for the Care and Use of Laboratory Animals* [National Institutes of Health (NIH), Bethesda, MD, USA].

Serum measurements

The endogenous PRL (rat PRL) in serum and milk was evaluated with ELISA (30). This assay has a limit of detection of 0.2 ng/ml; intra-assay and interassay coefficients of variation of 8.12 and 12.3%, respectively; and does not recognize ovine and human PRL. As controls to evaluate PRL levels in the milk, different concentrations of rat PRL were added to a fixed volume of milk. We observed that the milk did not alter the capacity of the antibodies to recognize PRL. The final dilutions of serum and milk used to detect PRL were 1:50 for dam's serum at d 7 and 21 of lactation, and 1:500 and 1:300 for milk at d 7 and 21 of lactation. Ovine PRL, used in osmotic pumps, was measured by using the Nb2 cell bioassay, a standardized method based on the proliferative response of Nb2 lymphoma cells to PRL, as previously described (31). This assay was made specific to ovine PRL by using anti-ovine PRL antibodies (National Hormone and Peptide Program, C-3581016789; Research Resource Identifier RRID AB_2629483) that do not cross-react with rat PRL. The difference in Nb2 bioactivity between the absence and presence of anti-ovine PRL quantified the levels of ovine PRL in rat serum and milk. The serum levels of insulin were quantified by using ELISA kits from Merck Millipore (Billerica, MA, USA). Hepatic triglyceride levels were measured by using a Colorimetric Assay Kit from Cayman Chemical (Ann Arbor, MI, USA).

Insulin tolerance test

ITT was performed in offspring on d 19 of lactation. For that purpose, pups were withheld food for 2 h, and blood glucose levels were measured in tail vein samples at 0, 15, 30, 60, 90, and 120 min after an intraperitoneal injection of 0.5 U/kg insulin (Humulin R; Eli Lilly, Indianapolis, IN, USA).

Milk sample collection

The milk sample collection procedure was based on the method described by DePeters *et al.* (32). Briefly, on d 7 and 21 of lactation, mothers were separated from their pups for 4 h (with the purpose of allowing the accumulation of milk in the MGs), and 30 min before collecting the milk they were injected intraperitoneally with 8 IU/kg of synthetic oxytocin (Oxitopisa; Pisa Farmaceutica, Jal, Mexico) to induce milk ejection. Lactating animals were then anesthetized with inhaled ether, and milk samples were collected by using a vacuum system. Milk samples were stored at −20°C for subsequent analysis.

Milk yield

Milk yield was measured on d 8 and 19 of lactation according to the method described by Sampson and Jansen (33). The pups of every litter were weighed in the morning (W1) and separated from their mothers for 4 h. Pups were then weighed (W2) and returned to their mothers for a suckling period of 1 h, and then weighed again (W3). Milk yield was calculated by W3 − W2 plus the weight loss caused by the metabolic processes in the pups during the suckling period (W2 – W1)/4. Thus, milk yield = (W3 − W2) + [(W2 − W1)/4].

MG, adipose tissue, and liver histology

The MG of lactating rats, subcutaneous adipose tissue (SAT) (inguinal), visceral adipose tissue (VAT) (epididymal), and liver

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tissue from offspring were collected on d 21 of lactation. Tissues were fixed in 10% formalin, sectioned (3 μm for liver, 5 μm for MGs, and 7 μm for adipose tissue) and stained with hematoxylin and eosin. For MG analysis, the mean parenchymal and adipose areas were quantified from 9 fields per animal in 6–13 lactating rats, and the ratio between parenchymal and adipose areas was determined. In SAT and VAT of the offspring, adipocyte size was determined by calculating the mean adipocyte area from 9 fields per animal in 7–20 pups, and the relative adipocyte number was calculated by dividing the fat pad weight by the mean adipocyte area in that fat depot. Representative images from liver sections were taken from each group. All the images were analyzed by using ImageJ software (NIH).

Quantitative RT-PCR

MG sections were obtained on d 21 of lactation, frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted by using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized by using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). PCR products were detected and quantified with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in a final reaction of 10 μl containing template and 0.5 μM of each primer pair for the respective rat genes: *Tnf*, *Tph1*, *Csn2*, *Lalba*, and *Prlr*. Amplification was performed in Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) using a protocol of 10 min of denaturation at 95°C, followed by 35 cycles of amplification (10 s at 95°C, 30 s at the primer pair–specific annealing temperature, and 30 s at 72°C). The gene expression in MG was normalized to the housekeeping gene *CypA*. Primer sequences used were: *CypA* (cyclophilin A) 5′-TGGGGAGAAAGGATTTGGCT-3′ (forward) and 5′-TTTGCCATGGACAAGATGCC-3′ (reverse); *Tnf* (TNF-α) 5′-GGGCTTGTCACTCGAGTTTT-3′ (forward) and 5′-TGCCTCAGCCTCTTCTCATT-3′ (reverse); *Tph1* (tryptophan hydroxylase 1) 5′- ACACCTGCCACGAACTCTTA-3′ (forward) and 5′-AGTGCATGTCTGAGCTCACT-3′ (reverse); *Csn2* (β-casein) 5′-TCTTGCAGTCCCATTCCACA-3′ (forward) and 5′-GCATCTGTTTGTGCTTGGGA-3′ (reverse); *Lalba* (α-lactalbumin) 5′-CTGCCTTTCAAGCCACAGAG-3′ (forward) and 5′-TGTTCTCTGACTCGGGGAAC-3′ (reverse); and *Prlr* (prolactin receptor) 5′-ATCTTCAACATGGCCATTAC-3′ (forward) and 5′-TTCTTCCTCTCCAGTCTCAA-3′ (reverse).

Western blot

Pulverized frozen liver sections from pups on postnatal d 21 were homogenized in lysis buffer (0.1 M Tris-HCl, 0.2 M EGTA, 0.2 M EDTA, 0.1 M sodium orthovanadate, 50 mM sodium fluoride, 100 mM sodium acid pyrophosphate, 250 mM sucrose, and pH 7.5) and protein concentrations quantified with Bradford reagent (Bio-Rad Laboratories). Sixty micrograms of liver protein were subjected to SDS/PAGE, blotted, and incubated overnight with 1:1000 anti–phospho-STAT5 (9351; Cell Signaling Technology, Danvers, MA, USA) or 1:500 anti-STAT5 (sc-1081; Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies. Blots were washed in Tris-buffered saline/Tween-20, and detection was performed by using goat anti-rabbit conjugated to horseradish peroxidase (1:5000) as secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Protein densities were quantified with Quantity 1 Software (Bio-Rad Laboratories).

Statistics

Statistical data analysis was performed by using Minitab 16 (Minitab, State College, PA, USA) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Differences between 3 or more groups were evaluated by using a 1-way ANOVA followed by the Tukey *post hoc* comparison test. The threshold for significance was set at a confidence interval of 95%.

RESULTS

Maternal overnutrition by HFD feeding during lactation

To evaluate whether an obesogenic diet during lactation would affect the levels and function of PRL, rats were fed an HFD or a CD from d 1 to 21 of lactation. Because our hypothesis was that an HFD would reduce the levels of serum PRL, rats given an HFD were implanted (HFD + PRL) or not (HFD) on lactation d 4 with a 28-d osmotic minipump delivering PRL to compensate for a possible reduction of PRL levels. Interestingly, exposure to HFD during lactation did not lead to higher body weight gain (BWG) in the dams, which is consistent with previous reports using long-term obesity models (34, 35). Rats on an HFD exhibited an unchanged body weight curve during the 21 d of lactation compared with animals fed a CD (**Fig. 1*A***). However, when BWG from d 1 to 21 of lactation was calculated, rats on an HFD had significantly reduced BWG compared with animals on a CD (Fig. 1*B*). Rats on an HFD + PRL exhibited a body weight curve that was almost identical to that of CD-fed animals, and their BWG was significantly higher than that of their HFD-fed counterparts. Regarding food consumption, rats on an HFD with or without PRL treatment showed reduced food intake in grams of pellets (Fig. 1*C*); however, given the higher caloric content of the HFD, animals on this diet displayed increased caloric intake compared with CD-fed animals. Surprisingly, HFD feeding during lactation did not lead to higher body weight in the dams but rather to lower BWG. Because HFD-fed animals consumed more calories than their CD-fed pairs, the energy surplus is likely utilized by their increase in energy expenditure (not measured in our experiments) or directed toward milk energetic composition, likely as FA. In support of the latter possibility, the BWG of the dams and their litter at the end of lactation was 11.5% higher in the HFD-fed group than in the CD-fed counterpart (Fig. 1*D*), whereas dam + litter BGW in the HFD + PRL group was not different from either CD- or HFD-fed animals. Accordingly, the litters from the HFD groups (with and without PRL treatment) showed higher BWG than those from CD dams (Fig. 1*E*).

PRL levels are reduced in the milk of HFD-fed lactating rats, in parallel with reduced milk yield

We next evaluated whether a maternal HFD feeding during lactation led to altered PRL levels in serum and milk, as well as milk yield at 2 time points of the lactation period: early lactation (d 7–8) and late lactation (d 19–21).

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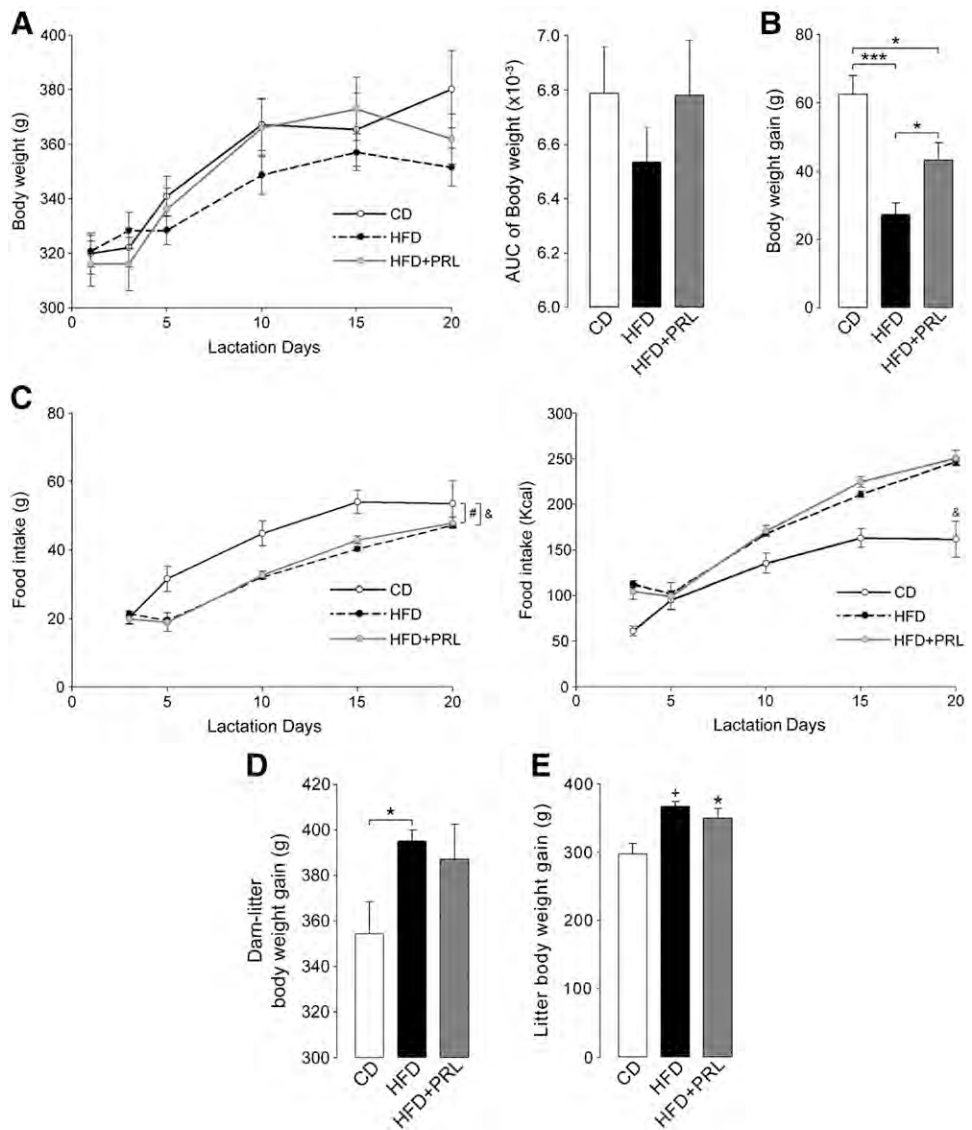

Figure 1. Effects of maternal intake of an HFD during lactation on the BWG and food consumption of the dams. Body weight (BW) and area under the curve (AUC) of body weight during lactation (*A*), BWG from d 1 to 21 of lactation (*B*), food intake in grams and kilocalories during lactation (*C*) in dams fed with CD or HFD in the absence or presence of osmotic minipumps delivering PRL to the mothers (HFD + PRL) during lactation (n = 6–13). Dam + litter BWG (*D*) and litter BWG (*E*) from d 1 to 21 of lactation (n = 6–13 dams and litters). Data are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.01$ *vs.* CD, $^{\&}P < 0.05$ *vs.* CD. Statistical differences were determined with 1-way ANOVA or repeated measures ANOVA (*C*), followed by the Tukey *post hoc* test.

Serum PRL levels in the mothers were not significantly altered by HFD feeding at either time point compared with mothers on a CD, although there was a trend (P = 0.14) toward lower PRL values in the HFD group on lactation d 7 (**Fig. 2*A***). As expected, serum PRL levels increased in HFD-fed rats implanted with PRL-loaded osmotic mini-pumps (HFD + PRL) at both time points.

Consistent with previous reports, PRL was highly concentrated in milk (36). Its levels were 4–6 times higher than those in the serum, ranging from 145 ± 22 ng/ml in the serum to 576 ± 68 ng/ml in the milk on lactation d 7, and from 26 ± 5 ng/ml in the serum to 152 ± 22 ng/ml in the milk on lactation d 21 (Fig. 2*A*, *B*). Interestingly, in HFD-fed rats, milk PRL levels were reduced by ~50% both on d 7 and 21 of lactation compared with the CD-fed counterparts (Fig. 2*B*). Conversely, when HFD-fed dams were treated with PRL, the levels of the hormone were nearly 6-fold higher and 2-fold higher compared with CD-fed animals on d 7 and 21 of lactation, respectively.

In parallel to the reduced PRL levels in milk, milk yield was 24% lower in HFD-fed mothers compared with CD-fed counterparts on d 8 of lactation, and this decrease was prevented by PRL treatment (Fig. 2*C*). On lactation d 19, the HFD did not decrease milk yield significantly, but PRL treatment did result in 22 and 34% higher milk yield compared with the CD and HFD groups, respectively. These findings show that an HFD-induced decrease in milk yield involves diminished PRL action in the MG that can be prevented by PRL treatment.

HFD feeding during lactation impairs MG structure and function, whereas PRL treatment prevents those alterations

Long-term obesity (before mating) is known to reduce lactation performance by inducing central and MG prolactin resistance (37, 38). However, it is unknown whether HFD feeding during lactation, independent of obesity, affects MG function and lactation efficiency. Histological evaluation of the MG showed that HFD feeding during lactation resulted in altered tissue structure, defined by increased MG weight (**Fig. 3*A***), decreased parenchymal area (Fig. 3*B*), increased adipose tissue deposits (Fig. 3*C*), and, therefore, a dramatic reduction in the MG parenchymal/

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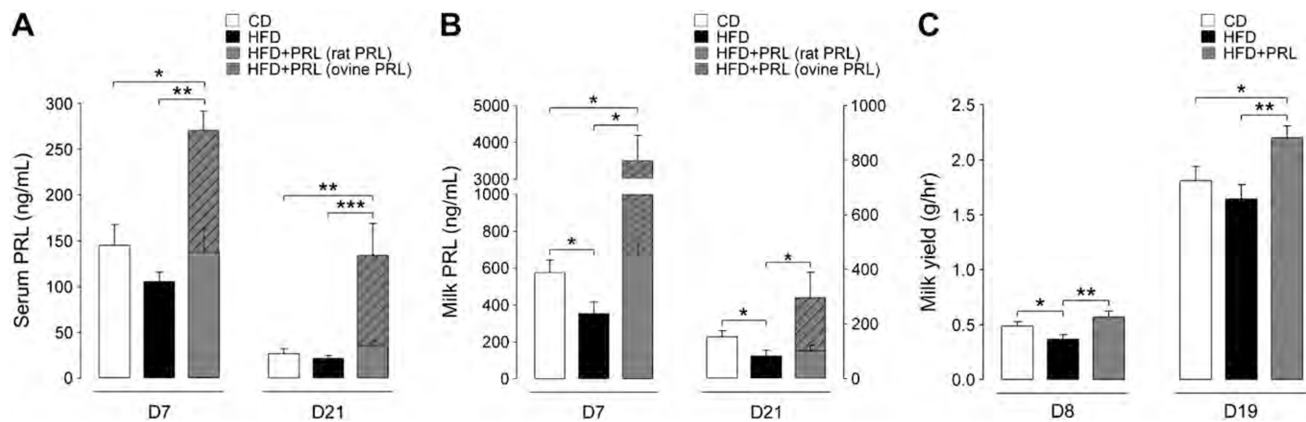

Figure 2. Maternal intake of an HFD during lactation reduces milk PRL levels and milk yield, whereas PRL treatment to lactating HFD-fed rats results in increased milk PRL levels and normalized milk yield. Serum PRL (*A*) and milk PRL (*B*) levels on d 7 and 21 of lactation in dams fed with a CD or an HFD in the absence or presence of osmotic minipumps delivering PRL to the mothers (HFD + PRL) during lactation (n = 5–7). Bars without lines are rat PRL (endogenous) levels quantified by radioimmunoassay; lined bars are ovine PRL levels delivered by pumps and measured by using the Nb2 assay. *C*) Milk yield on d 8 and 19 of lactation of dams fed with CD or HFD in the absence or presence of osmotic minipumps delivering PRL (HFD + PRL) during lactation (n = 40–48). Data are means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Statistical differences were determined with 1-way ANOVA, followed by the Tukey *post hoc* test.

adipose tissue ratio (Fig. 3*D, E*). In addition, reduced milk yield (Fig. 2*C*) alterations in MG function induced by the HFD feeding were observed at the gene expression level. HFD-fed dams exhibited reduced expression of the milk proteins β-casein and α-lactalbumin, reduced expression of the PRL receptor, and increased expression of the proinflammatory cytokine TNF-α and of tryptophan hydroxylase 1, the rate-limiting enzyme in serotonin synthesis known to be involved in MG involution (39, 40). Most of the observed alterations in the MG induced by the HFD feeding during lactation are consistent with those seen in models of long-term obesity (3, 41, 42). Interestingly, PRL treatment during lactation prevented most of the alterations induced by the HFD feeding. HFD + PRL–treated dams exhibited normalized parenchymal and adipose tissue areas, and therefore an improved parenchymal/adipose tissue ratio (Fig. 3*B, E*) and normalized gene expression levels (Fig. 3*F*). These results show that HFD-induced alterations in MG structure and function involve the impairment of PRL actions in the MG, as they can be prevented by PRL treatment.

HFD feeding in lactating dams results in increased body weight and adiposity of their offspring that are counteracted by PRL treatment

Our data confirm previous findings showing that maternal HFD feeding during lactation in rodents results in metabolic alterations in the offspring (5, 6, 8, 43). From postnatal d 1 to 21, offspring from HFD-fed mothers gained 23% more body weight than those from CD-fed dams (**Fig. 4*A***); this outcome was accompanied by a 2.5-fold increase in the VAT and a 1.7-fold increase in the SAT depots (Fig. 4*B, C*). The type of adipose tissue expansion was depot specific; VAT was characterized by increased adipocyte size (hypertrophy) and number (hyperplasia) (Fig. 4*D, E*), whereas SAT showed hypertrophy of the adipocytes but no change in adipocyte number (Fig. 4*F, G*). When the HFD-fed mothers were treated with PRL throughout lactation, their offspring exhibited a small but significant 4.6% decrease in BWG compared with those from HFD-fed mothers, as well as decreased adiposity in the VAT but not in the SAT. PRL had no effect on adipocyte hypertrophy but resulted in depot-specific effects in adipocyte hyperplasia. In VAT, PRL treatment to the mothers prevented adipocyte hyperplasia in the offspring, whereas in SAT adipocyte numbers did not change between offspring from HFD-fed mothers with and without PRL treatment. However, compared to offspring from CD-fed mothers, PRL treatment in HFD-fed mothers increased adipocyte hyperplasia in SAT.

To test whether the beneficial effects on the offspring of increasing PRL levels in their mothers were due to PRL preventing the HFD-induced alterations in MG morphology and function, or due to the transfer of PRL into the milk and to its direct actions on the offspring, we treated a group of pups from HFD-fed mothers with PRL *via* oral administration. Oral delivery of PRL to the offspring resulted in increased PRL levels in their serum (**Fig. 4*H***), similar to the levels observed in the serum of offspring from HFD-fed mothers treated with PRL. Oral PRL did not alter the body weight of offspring from HFD-fed mothers, as they gained as much weight as the vehicle-treated offspring (Fig. 4*A*). However, even though their BWG was the same, the PRL-treated offspring showed significantly reduced visceral adiposity (Fig. 4*B*) and increased adipocyte hyperplasia in SAT (Fig. 4*G*), supporting that milk PRL acts directly on the offspring to reduce maternal HFD-induced visceral adiposity.

PRL treatment to mothers prevents maternal HFD–induced fatty liver in their offspring

A maternal HFD during lactation also led to increased hepatic triglyceride accumulation in the offspring, as evidenced by >2-fold higher triglyceride levels in the

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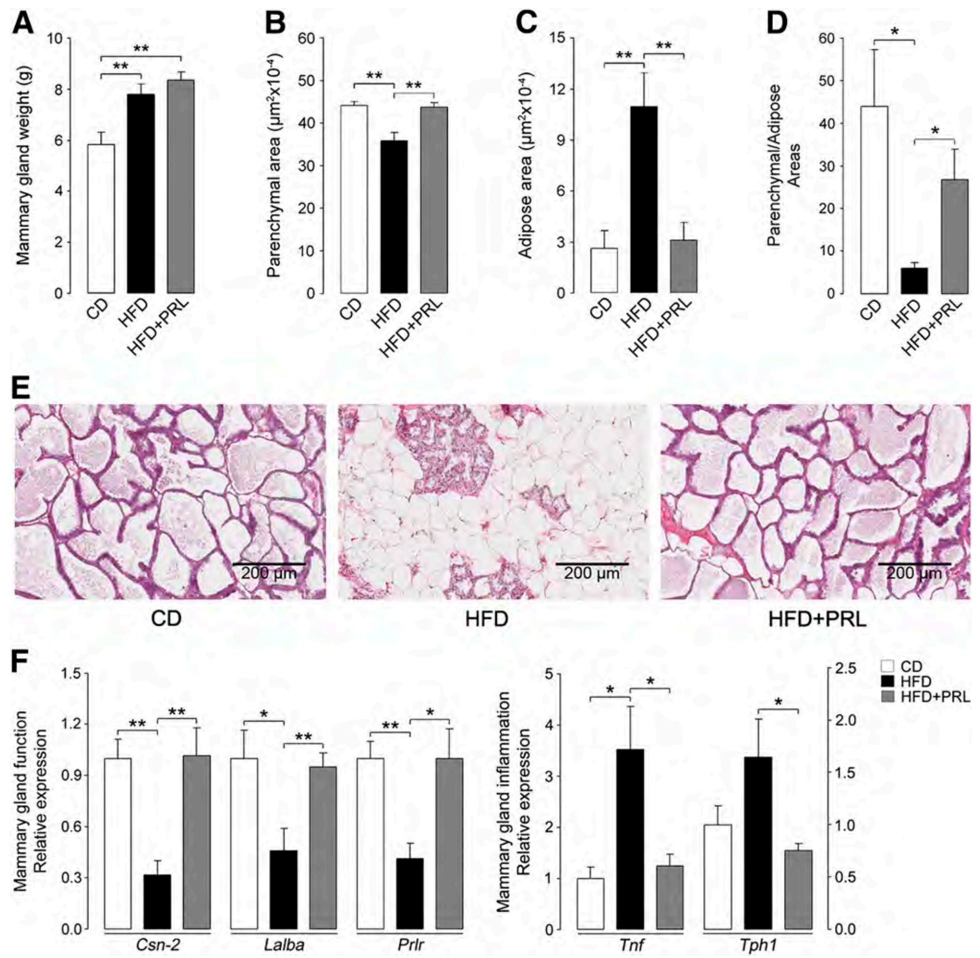

Figure 3. Maternal intake of an HFD during lactation leads to structural and functional alterations in the MG, and PRL treatment to the mothers prevents such alterations. Weight (*A*), parenchymal area (*B*), adipose area (*C*), ratio of parenchymal to adipose area (*D*), and representative sections stained by hematoxylin and eosin (n = 6–13) (*E*) of MGs. *F*) Gene expression analysis by qPCR of the genes related to MG function, β-casein (*Csn2*), α-lactalbumin (*Lalba*), and PRL receptor (*Prlr*); and of the genes related to MG inflammation and involution, TNF-α (*Tnf*), and tryptophan hydroxylase 1 (*Tph1*) (n = 6–11). All evaluations were made on d 21 of lactation in MG from dams fed with a CD or an HFD in the absence or presence of osmotic minipumps delivering PRL to the lactating mothers (HFD + PRL). Data are means ± SEM. $*P < 0.05$, $**P < 0.01$. Statistically significant differences were determined with 1-way ANOVA, followed by the Tukey *post hoc* test.

livers of offspring nursed by HFD-fed mothers compared with those from CD-fed dams (**Fig. 5*A*, *B***). However, PRL treatment to HFD-fed mothers prevented the development of fatty liver in their pups, as seen by normalized liver triglyceride levels and less appearance of fat stores in liver histologic sections, compared with offspring nursed by HFD-fed mothers in the absence of PRL. Oral PRL treatment to the offspring showed a nonsignificant reduction in liver triglyceride levels but evident reduced fat deposits in liver histologic sections. Triglyceride levels in the PRL-treated offspring did not differ from those of their pairs from CD-fed mothers.

PRL treatment in mothers or offspring rescues STAT5 phosphorylation in the liver, blunted by maternal HFD feeding during lactation

To evaluate whether PRL treatment in mothers or offspring was acting on PRL target tissues of the pups, we evaluated the phosphorylation of STAT5, a canonical signaling transcription factor downstream of the PRL receptor, in livers from offspring on d 21 of lactation. Liver was used because this tissue has one of the highest expression of PRL receptors (44); it is a relevant metabolic tissue and a direct target of PRL actions (45). We observed that STAT5 phosphorylation decreased in livers of pups from HFD-fed dams compared with those from CD-fed mothers, suggesting that even if serum PRL levels are similar in the pups from both groups (Fig. 4*H*), there is PRL resistance in those from HFD-fed dams. On the contrary, we observed that PRL treatment, either in HFD-fed dams or directly in their offspring, restored liver STAT5 phosphorylation levels (Fig. 5*C*, *D*).

PRL treatment in mothers or offspring ameliorates hyperinsulinemia and insulin resistance in the offspring induced by maternal HFD during lactation

Maternal HFD feeding during lactation resulted in hyperinsulinemia in their offspring (**Fig. 6*A***): serum insulin levels increased 73% in the pups from HFD-fed mothers compared with their pairs from CD-fed dams. Moreover, offspring from HFD-fed mothers developed marked insulin resistance at weaning, as seen by a dramatically reduced insulin-induced glucose lowering effect in ITTs (Fig. 6*B*). When HFD-fed mothers were treated with PRL throughout lactation, their offspring showed reduced insulin levels that were close to those from the offspring of CD-fed dams and improved insulin sensitivity in the ITT. Conversely, oral PRL treatment to the offspring did not reduce the insulin levels in the pups from HFD-fed mothers but did improve their insulin sensitivity at levels similar to those observed in the offspring from PRL-treated mothers.

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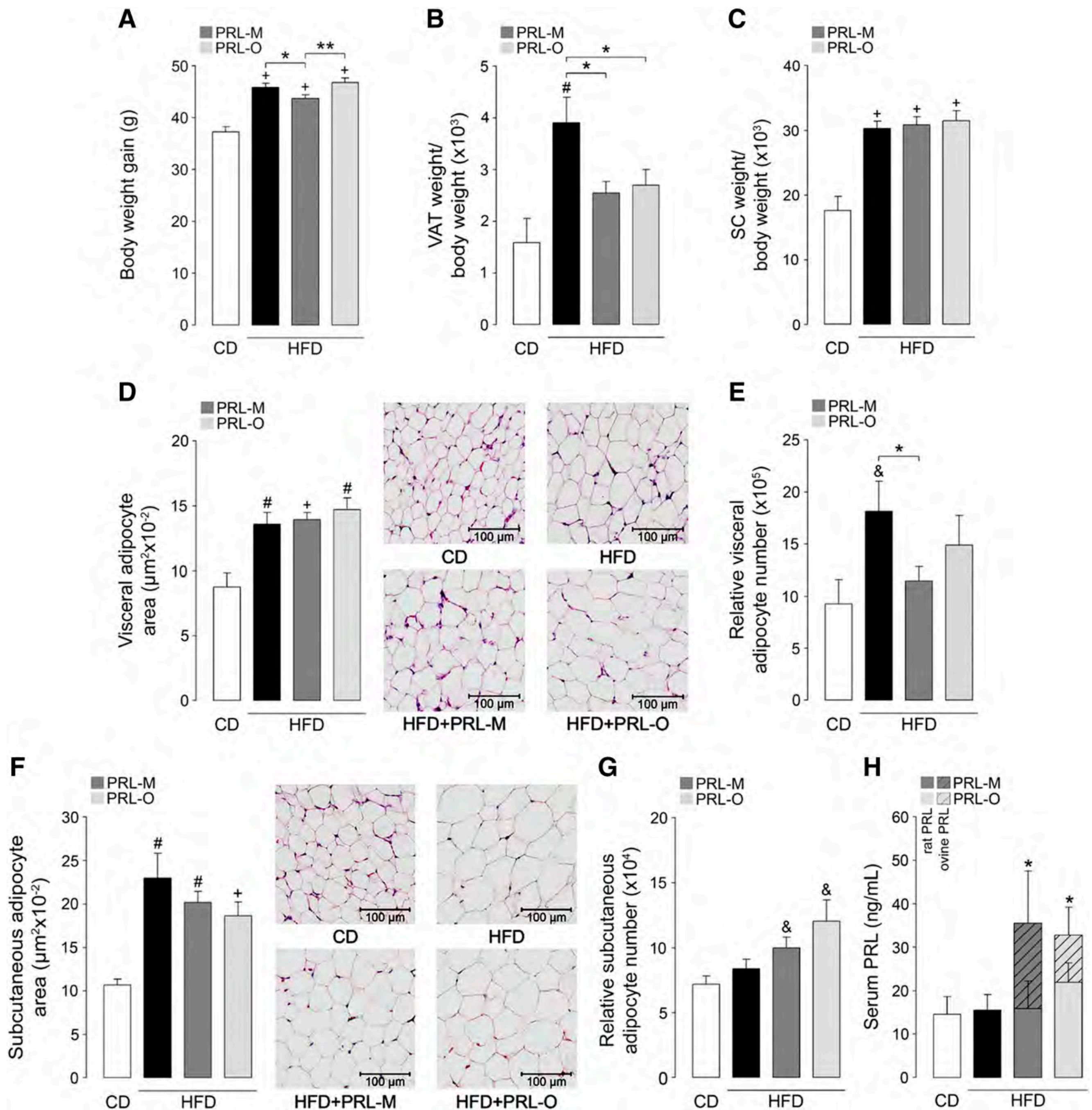

Figure 4. Maternal intake of an HFD during lactation promotes the development of obesity and increased adiposity in the offspring at weaning, and PRL treatment to the mothers or directly to the offspring prevents such alterations. BWG (*A*), VAT (*B*), and SAT (*C*) weight normalized to body weight (n = 11–20), VAT adipocyte area (*D*) and number (*E*), SAT adipocyte area (*F*) and number (*G*), and serum PRL levels (n = 8–11) (*H*). Evaluations were made on d 21 of lactation in offspring from dams fed with a CD or with an HFD, that received or not maternal PRL treatment (PRL-M) by subcutaneous osmotic minipumps, or offspring PRL treatment (PRL-O) by oral administration during lactation. Tissue areas were evaluated in 9 fields per adipose tissue of 7–20 animals per group, and images are representative sections of adipose tissue stained with hematoxylin and eosin to show adipocyte size; relative adipocyte number per fat depot are from 7–20 animals. *H*) Bars without lines are rat PRL (endogenous) levels quantified by radioimmunoassay, and lined bars are ovine PRL levels delivered by pumps and measured by using the Nb2 assay. Data are means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^+P < 0.001$ *vs.* CD, $^\#P < 0.01$ *vs.* CD, $^\&P < 0.05$ *vs.* CD. Statistical differences were determined with 1-way ANOVA, followed by the Tukey *post hoc* test.

DISCUSSION

During lactation, maternal metabolic disturbances resulting from obesity or overnutrition lead to altered milk composition and metabolic dysfunction in the offspring (3–8, 46). However, the nature of the factors altered in the milk damaging the metabolic programming from mothers to offspring is poorly understood. The present article shows that the adverse metabolic effects on the offspring caused by an HFD exposure to their lactating mothers involve, on

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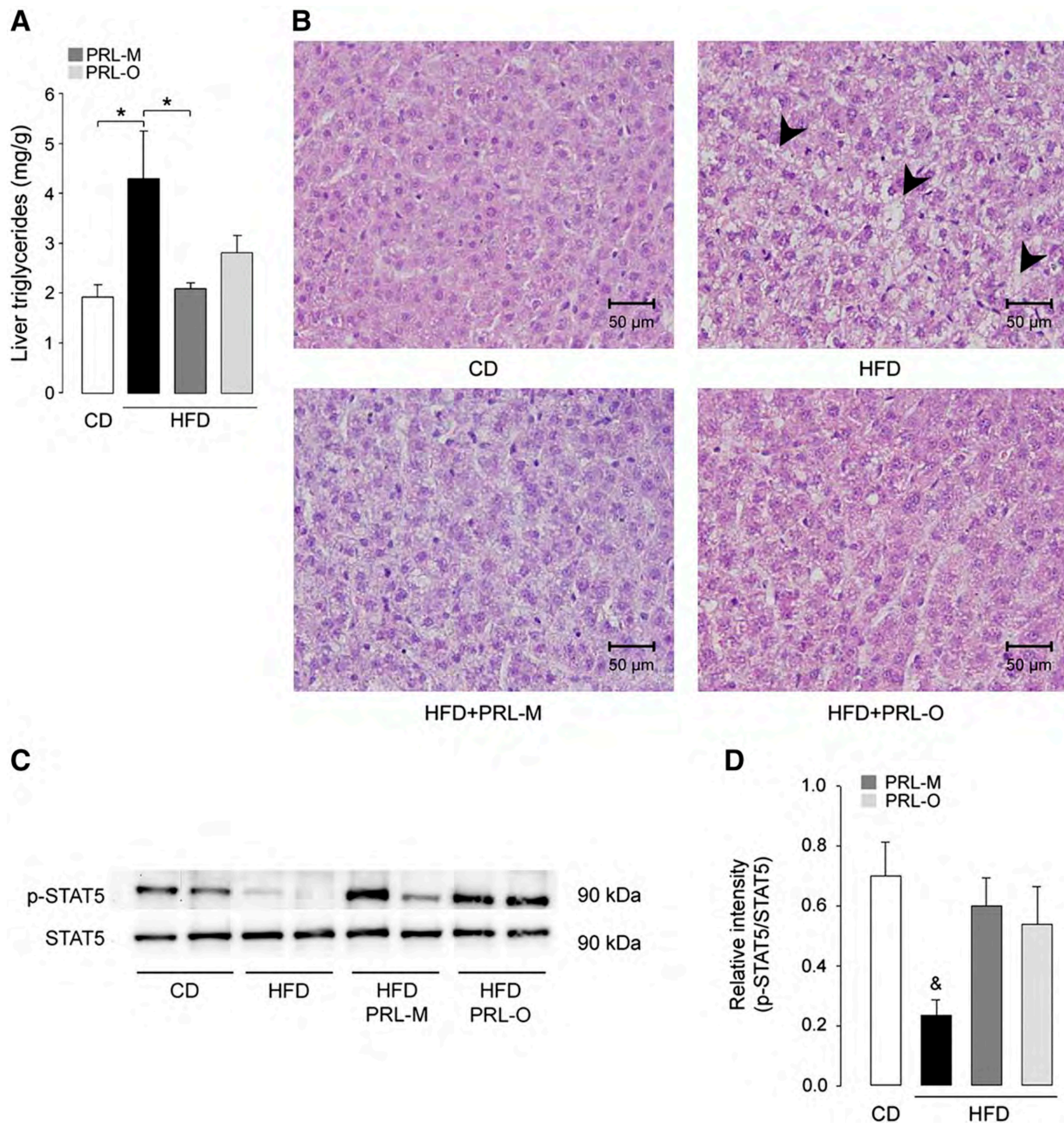

Figure 5. PRL treatment prevents liver triglyceride accumulation and prevents liver PRL resistance in offspring from HFD-fed mothers during lactation. Liver triglyceride content (n = 10–12) (*A*), representative sections of liver stained by hematoxylin and eosin showing accumulation of lipid droplets (*B*), representative Western blot for pSTAT5 and STAT5 in livers (*C*), and quantification of pSTAT5/STAT5 by densitometry (n = 8–9) (*D*) from 21-d-old offspring from dams fed with a CD or with an HFD that received or not maternal PRL treatment (PRL-M) by subcutaneous osmotic mini-pumps or offspring PRL treatment (PRL-O) by oral administration during lactation. Data are means ± SEM. $^{*}P < 0.05$, $^{\&}P < 0.05$ HFD *vs.* all the groups. Statistical differences were determined with 1-way ANOVA, followed by the Tukey *post hoc* test.

the one hand, impaired PRL actions at the MG level and, therefore, in the yield and composition of milk; and on the other hand, reduced PRL levels in the milk, leading to diminished beneficial effects of the hormone on the offspring.

In humans, obesity is associated with reduced breastfeeding success, delayed initiation of lactation, lower milk yield, and early cessation of breastfeeding (37, 38, 47, 48). Consistently, in rodents, an HFD before mating and throughout gestation and lactation results in altered MG morphology and function as well as reduced lactation performance (37, 41, 42). Mechanisms involved in these alterations include diminished PRL responses to sucking (49), increased serotonin production in the MG leading to inflammation (42), and PRL resistance both in the hypothalamus and in the MG, evidenced by elevated basal phosphorylated STAT5 levels in the MG of obese lactating mice that do not respond further to an acute PRL injection (37). Our model of HFD only during lactation allows for the selective evaluation of the effects of an obesogenic diet during this critical time frame, without confounding factors derived from diet-induced alterations occurring during gestation. We found that even when MG development during gestation occurs in a normal

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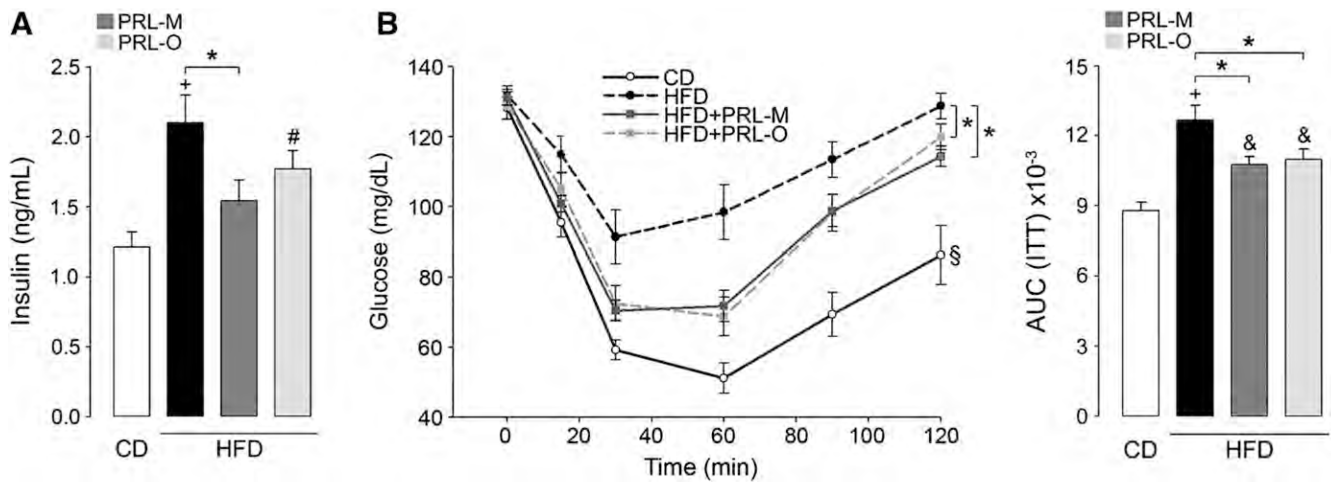

Figure 6. Maternal intake of an HFD during lactation results in hyperinsulinemia and insulin resistance in their offspring, and PRL treatment to the mothers or directly to the offspring prevents these metabolic disturbances. Serum insulin levels ($n = 10–21$) (*A*) and ITT and area under the curve of ITT ($n = 11–22$) (*B*) evaluated on d 21 of lactation in offspring from mothers fed with a CD or with an HFD, that received or not maternal PRL treatment (PRL-M) by subcutaneous osmotic minipumps, or offspring PRL treatment (PRL-O) by oral administration during lactation. Data are means ± SEM. $^{*}P < 0.05$, $^{+}P < 0.001$ *vs.* CD, $^{\#}P < 0.01$ *vs.* CD, $^{\&}P < 0.05$ *vs.* CD, § *vs.* all the groups. Statistical differences were determined with 1-way ANOVA or repeated measures ANOVA (*B*), followed by the Tukey *post hoc* test.

dietary environment, an HFD challenge during lactation replicates most of the alterations observed in the MG in models of long-term obesity (41, 42), suggesting that lactation is the most sensitive window for alterations in MG development and function.

We found that impaired MG structure and function induced by an HFD derives mainly from decreased PRL effects in the gland. This outcome may be due to PRL resistance, as proposed recently by Buonfiglio *et al.* (37), but also to a lower sensitivity of the MG to PRL because the HFD reduces the MG expression of the PRL receptor. These two mechanisms are not mutually exclusive because reduced PRL receptor levels could mediate PRL resistance. However, unlike the classic concept of hormonal resistance leading to higher hormonal levels in the circulation (*i.e.*, hyperinsulinemia and hyperleptinemia) (50, 51), HFD does not increase systemic PRL levels. Thus, PRL insensitivity may be a more likely mechanism in our model. It remains to be determined whether serum PRL concentrations are altered in long-term obesity conditions during reproductive stages.

The fact that the HFD-induced alterations in the MG are prevented by PRL treatment supports the theory that impaired lactation performance due to obesity and overnutrition involves diminished PRL actions and suggests that PRL treatment may be used to improve lactation outcomes in obese women. In this regard, PRL treatment has been used successfully in cases of lactation insufficiency with or without PRL deficiency (52). The mechanisms that mediate PRL protection against MG morphologic and functional alterations induced by HFD feeding during lactation involve canonical PRL effects; that is, promotion and maintenance of MG differentiation and function (19, 53, 54) and prevention of the early MG involution induced by the obesogenic diet (3, 41, 42). The finding that endogenous PRL levels in the milk of HFD + PRL animals are similar to controls, whereas levels are reduced in dams fed an HFD at d 7 of lactation, may reflect the fact that by preventing HFD-induced MG alterations, PRL treatment allows the adequate transfer of PRL from serum to milk, and it may even stimulate such transfer.

Another strategy to elevate PRL levels in obese mothers may be to increase dietary protein intake. Low protein diets (5–10% of total caloric intake) reduce PRL serum levels in males and in gestating and lactating rodents (55–58). Conversely, a high protein diet during gestation and lactation increases PRL serum levels (58), and PRL promotes amino acid transport in the MG during lactation. In this study, we used normal protein diets (CD = 28% and HFD = 20%) and in agreement observed no significant changes in PRL serum levels in CD *vs.* HFD-fed animals and no alterations in body composition of the dams or the offspring that could suggest low protein intake–induced undernutrition; in addition, normal protein levels were observed in the milk from HFD-fed dams compared with controls (Supplemental Fig. 1*A*–*G*). Therefore, it is unlikely that the deleterious effects of the HFD on dams and their offspring are due to insufficient dietary protein. However, whether increasing dietary protein intake in obese lactating mothers improves lactation outcomes and offspring's metabolic health is worth investigating.

Maternal obesity results in multiple alterations, most of them unknown, in milk composition, including changes in the lipid profile and in hormone and cytokine levels. Milk from obese humans and rodents has a higher content of long-chain saturated FA and higher levels of insulin and leptin (3, 7, 8, 46, 59–62). PRL is an important regulator of milk composition; it promotes the expression of β-casein (63), whey acidic protein (64), lactoglobulin (65), and α-lactalbumin (66), leading, in turn, to increased lactose biosynthesis and milk volume (likely through enhanced production of lactose) (52, 67). PRL also increases calcium levels (68, 69) and reduces sodium concentrations (69), at

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least in part secondary to its effects, reducing the permeability of the epithelial barrier of the MG (69, 70). Two groups of milk components that could explain indirect beneficial actions of PRL on the offspring metabolism are FA and milk oligosaccharides. PRL increases MG lipid levels by regulating the expression of proteins involved in FA synthesis, acetyl-CoA carboxylase, FA synthetase, acetyl-CoA synthetase, and pyruvate dehydrogenase (71–73). Many FA contained in milk have functional properties, and the best characterized are docosahexaenoic acid, arachidonic acid (9, 11), and ω-3 and ω-6 polyunsaturated FA (74). However, there is a great diversity of FA moieties, such as the newly discovered mammalian branched FA esters of hydroxy FA, which have antidiabetic and anti-inflammatory properties (75). Whether FA esters of hydroxy FA or other novel functional FA are present in breast milk, and whether their levels are modulated by PRL action in the MG during lactation, is unknown. PRL also elevates the levels of neutral and acidic oligosaccharides in the milk (69). This group of glycans serves as prebiotics for intestinal commensal bacteria and also interacts with pathogenic bacteria, preventing their binding to the host intestinal cells and therefore protecting against infections. Moreover, milk oligosaccharides may also act as immunomodulators to regulate the development of the infant intestine (69, 76, 77). Therefore, it is clear that by regulating MG function and milk composition, PRL regulates the offspring metabolism. Studying the molecular entities in the milk that mediate PRL effects in the offspring warrants further investigation.

Although PRL is known to be present in the milk in high quantities (24, 25), whether milk PRL levels are altered by maternal overnutrition was not previously investigated, to the best of our knowledge. We found that an HFD feeding during lactation results in reduced milk PRL levels. Interestingly, maternal serum PRL levels were not significantly altered by the HFD, although there was a trend toward lower levels on lactation d 7 ($P = 0.14$), suggesting that decreased levels of the hormone in the milk are mainly due to its reduced transfer from the circulation to the milk. This theory is supported by our findings showing that PRL expression in the MG is very low and is not altered by the diet (data not shown), arguing that most, if not all, of the PRL present in the milk derives from the circulating pool. PRL transfer from serum to milk is mediated by PRL receptor-dependent endocytosis (78, 79). Consistent with these findings, we observed that in response to an HFD, milk PRL levels change in parallel with PRL receptor expression in the MG. Both are reduced in lactating rats fed an HFD and normalized to control levels by PRL treatment, supporting the theory that HFD-induced reduction in PRL receptor levels in the MG contribute to decreased PRL levels in the milk. A relevant question that remains to be addressed is what drives HFD-induced PRL receptor down-regulation in the MG?

There is extensive evidence supporting that once in the milk, PRL is transported from the gastrointestinal tract to the circulation of the lactating offspring (26, 80). This action has been shown to occur specifically by jejunum and ileum transportation through a transepithelial pathway (80). Radiolabeled PRL administered to the lactating mother is found in the serum of pups after suckling (26). Interestingly, PRL levels are very low in the circulation of newborn rodents during the first 2 wk of lactation (81). However, PRL bioactivity increases in the neonate circulation after suckling (25). In agreement with milk PRL being able to reach the offspring circulation, we used the highly sensitive Nb2 bioassay (31) and detected the bioactive ovine PRL used in the osmotic mini-pumps implanted into the mothers, or given directly to the offspring orally, in the serum of the pups.

The evidence supporting the theory that milk PRL regulates the neuroendocrine, immune, and reproductive systems in neonates (16, 29, 36) arises from experiments in which the dopamine receptor agonist bromocriptine has been used to reduce PRL levels in the serum and therefore in the milk of lactating rodents. However, it is unclear whether the effects seen in the offspring from bromocriptine-treated mothers are due to effects on the MG and milk composition resulting from reduced PRL levels in the mother, or to effects of bromocriptine itself transferred to the milk, rather than to direct effects of milk PRL on the offspring (82, 83). Our finding that treating the pups directly with PRL results in improved metabolic outcomes in the offspring shows for the first time a direct action of milk PRL in the nursing young.

Other hormones present in milk such as leptin and FGF21 have been shown to exert direct actions on the offspring (14, 84). Leptin intake during lactation in rat offspring reduces food intake and lowers body weight and adiposity when animals are adult, even if they are given an HFD (85). In addition, oral intake of FGF21 in lactating pups induces the expression of intestinal hormones and digestive enzymes, and these effects also occur when FGF21 null pups nurse from wild-type dams (14). The case of PRL is particularly interesting because this hormone is one of the reportedly few hormones with higher levels in the milk than in the maternal circulation (14, 86–88). Once in the offspring circulation, milk PRL may exert direct actions on different PRL target metabolic tissues, such as adipose tissue, liver, and pancreas (23, 45, 89). In adults, these organs express PRL receptors (54, 90), although it is unknown whether PRL receptors are already present in such organs in the lactating offspring. The effects of milk PRL in the offspring's adipose tissue are supported by our findings showing that oral PRL treatment to the neonatal offspring from HFD-fed mothers results in reduced VAT adiposity and increased subcutaneous adipocyte hyperplasia. In agreement, we have shown that in adult obese rats, PRL treatment promotes adipocyte hyperplasia while reducing visceral adipocyte hypertrophy (23).

Milk PRL may also act directly in the offspring liver. Although offspring from HFD-fed mothers exhibited increased liver triglyceride levels compared with those from CD-fed mothers, oral PRL treatment to offspring from HFD-fed mothers showed no significant difference in triglyceride levels compared with control pups or to pups from HFD-fed dams. This observation suggests that oral PRL had some effect in the liver, which

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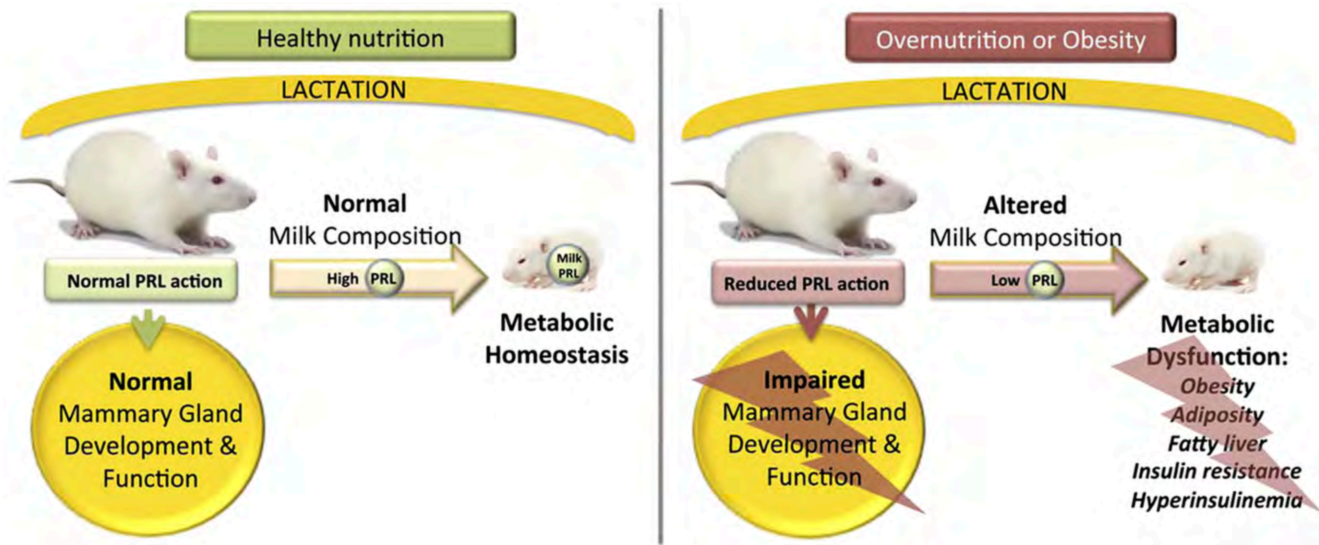

Figure 7. HFD feeding to the mothers during lactation impairs maternal and offspring PRL actions to negatively alter the metabolism of the nursing young. A healthy nutrition favors PRL's beneficial effects on MG development and function and adequate milk composition, including high levels of PRL in milk to maintain offspring metabolic homeostasis (left panel). Overnutrition or obesity during lactation impairs PRL actions and results in reduced milk yield, altered milk composition, including lower levels of PRL in milk and, thereby, metabolic dysfunction of the offspring (right panel).

was also evident by reduced appearance of fat deposits in histologic sections of the tissue. In adult animals, PRL stimulates liver insulin sensitivity. Knockdown of the liver PRL receptor leads to insulin resistance, whereas overexpression of the PRL receptor promotes insulin sensitivity, and the PRL effect is mediated by STAT5, a canonical PRL signaling molecule (45). Additional support for the direct actions of milk PRL in the offspring derive from the finding that PRL treatment in the offspring or in the mothers increases STAT5 phosphorylation in the liver of the offspring, whereas its phosphorylation is reduced in offspring from HFD-fed dams compared with offspring from CD dams. This finding suggests that even if the offspring from HFD-fed dams have normal PRL serum levels, they develop PRL resistance, and this resistance can be counteracted by PRL treatment. It remains to be determined whether PRL resistance in the offspring induced by the maternal obesogenic diet extends to tissues other than the liver and if it is due to reduced PRL receptor expression (as seen in the MG of the HFD-fed mothers) or rather to altered PRL receptor signaling. Notably, oral PRL ameliorated insulin resistance in the offspring from HFD-fed dams. This effect may result from improved adipose tissue and liver function but also from PRL effects on other metabolic tissues such as muscle, pancreas, hypothalamus, and the intestine, all organs known to play important roles in glucose and insulin homeostasis (91).

Our results in a model of maternal HFD feeding during lactation, using PRL treatment either to the mother to compensate for the reduced PRL actions in the MG or directly to the offspring to restore the low PRL levels seen in the milk, allowed us to uncover previously unappreciated roles of maternal PRL in regulating the metabolism of nursing young. Some of the beneficial effects of PRL in the offspring from HFD-fed dams seem to be primarily due to direct actions of milk PRL in the offspring, including reduced visceral adiposity and ameliorated insulin resistance, because these effects are of equal magnitude when PRL is given to the mother or to the offspring. Other PRL actions in the offspring are likely secondary to the effects of the hormone on the MG and milk composition, and therefore independent from milk PRL, such as the reduced BWG of the offspring seen when PRL was given to the mother but not when it was administered directly to the offspring. Finally, some effects are probably due to combined direct and indirect maternal PRL actions. One such example is the case of reduced liver triglyceride accumulation and lowered circulating insulin levels, as oral PRL in the offspring exerted partial effects reducing triglyceride and insulin levels, but those effects were of higher magnitude when PRL was given to the mother.

In summary, maternal PRL plays a key role in promoting neonatal offspring metabolism during lactation *via* indirect effects on the mother and direct effects on the nursing young. PRL acts on the MG to promote differentiation, enhance milk production, and determine milk composition; PRL reaches the nursing young by accumulating in milk to maintain their metabolic homeostasis. During lactation, the deleterious effects of maternal HFD feeding on offspring metabolism involve impaired PRL actions on both levels (**Fig. 7**). Our results suggest the potential therapeutic value of hyperprolactinemia-inducing drugs to treat obese women to help improve the metabolic health status of their offspring. FJ

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AUTHOR CONTRIBUTIONS

E. A. de los Ríos and Y. Macotela designed the research; E. A. de los Ríos, X. Ruiz-Herrera, V. Tinoco-Pantoja, and F. López-Barrera performed the research; E. A. de los Ríos, X. Ruiz-Herrera, and Y. Macotela analyzed the data; G. Martínez de la Escalera and C. Clapp discussed data, provided scientific expertise, and contributed to writing the manuscript; and Y. Macotela conceived and directed the study, and wrote the manuscript.

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Prolactin Promotes Adipose Tissue Fitness and Insulin Sensitivity in Obese Males

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Excessive accumulation of body fat triggers insulin resistance and features of the metabolic syndrome. Recently, evidence has accumulated that obesity, type 2 diabetes, and metabolic syndrome are associated with reduced levels of serum prolactin (PRL) in humans and rodents, raising the question of whether low PRL levels contribute to metabolic dysfunction. Here, we have addressed this question by investigating the role of PRL in insulin sensitivity and adipose tissue fitness in obese rodents and humans. In diet-induced obese rats, treatment with PRL delivered via osmotic mini-pumps, improved insulin sensitivity, prevented adipocyte hypertrophy, and reduced inflammatory cytokine expression in visceral fat. PRL also induced increased expression of *Pparg* and *Xbp1s* in visceral adipose tissue and elevated circulating adiponectin levels. Conversely, PRL receptor null mice challenged with a high-fat diet developed greater insulin resistance, glucose intolerance, and increased adipocyte hypertrophy compared with wild-type mice. In humans, serum PRL values correlated positively with systemic adiponectin levels and were reduced in insulin-resistant patients. Furthermore, PRL circulating levels and PRL produced by adipose tissue correlated directly with the expression of *PPARG, ADIPOQ,* and *GLUT4* in human visceral and sc adipose tissue. Thus, PRL, acting through its cognate receptors, promotes healthy adipose tissue function and systemic insulin sensitivity. Increasing the levels of PRL in the circulation may have therapeutic potential against obesity-induced metabolic diseases. **(*Endocrinology* 158: 56–68, 2017)**

Adipose tissue dysfunction is a hallmark of obesity-induced metabolic disorders and is also a feature of type 2 diabetes (T2D) and accelerated cardiovascular disease. Adipose tissue functions as an energy reservoir that is central for maintaining whole-body glucose and lipid homeostasis. In obesity, excessive lipid accumulation in adipocytes triggers mechanisms that result in systemic insulin resistance, including impaired adipogenesis (1), altered adipokine secretion, inflammation, fibrosis, reduced angiogenesis, excess free fatty acid release, and ectopic fat deposition (2, 3). Visceral adipose tissue (VAT) expansion is particularly associated with increased risk of metabolic disorders, whereas sc adipose tissue (SAT) accumulation has little or no negative metabolic impact and may even be protective (4, 5). Identifying factors that promote adipose tissue fitness may be of therapeutic value against insulin resistance and its associated metabolic diseases. Here, we suggest that one such factor is the hormone prolactin (PRL).

PRL is a multifunctional hormone with effects on metabolism. Increased PRL levels can stimulate food intake by promoting leptin resistance (6–10) and enhance body weight and adiposity (6, 7, 11–14) in rats and mice. Some of these actions were evaluated in females and may

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Abbreviations: Ccl2, chemokine (C-C motif) ligand 2; CD, control diet; BMI, body mass index; GTT, glucose tolerance test; HFD, high-fat diet; HOMA-IR, homeostasis model of insulin resistance; ITT, insulin tolerance test; Nb2, Noble rat lymphoma; PPARg, peroxisome proliferator-activated receptor-γ; PRL, prolactin; qPCR, Real Time-quantitative PCR; RXR, Retinoid X receptor; SAT, subcutaneous adipose tissue; T2D, type 2 diabetes; UNAM, Universidad Nacional Autónoma de México; VAT, visceral adipose tissue; Xbp1s, spliced form of X-box-binding protein-1.

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not occur in males (14, 15), consistent with the concept that they are part of the metabolic adaptations occurring during pregnancy and lactation, when high PRL levels, leptin resistance, hyperphagia, increased body weight, and adiposity coincide to increase nutrient availability to favor offspring development and survival (16). However, PRL can have an adverse impact on metabolism in scenarios of pathological hyperprolactinemia (prolactinomas). Studies, in both men and women, show that the occurrence of prolactinomas associates with higher prevalence of obesity and metabolic alterations such as dyslipidemia, glucose intolerance, and insulin resistance, and these alterations are reduced by lowering PRL levels with dopamine agonists (17–21). However, dopamine agonists may also increase body weight in patients with prolactinomas (21, 22), and there are studies in which body mass index (BMI) and metabolic improvement do not directly correlate with the reduction in PRL levels (23–25). In support of high systemic PRL being deleterious for metabolic homeostasis, PRL levels above 200 ng/mL and up to 4000 ng/mL in female mice for several months correlate with increased food intake, higher body weight and adiposity, glucose intolerance, and insulin resistance (14, 26).

PRL operates at different levels to regulate metabolism. It stimulates β-cell proliferation, survival, and insulin secretion in rodents and humans (27, 28) and promotes liver insulin sensitivity in rodents (29). PRL receptors are expressed on adipocytes from humans, rats, and mice, and PRL is produced by human adipose tissue (30), where it contributes to adipogenesis and inhibits lipolysis (31-33). PRL stimulates also the formation and function of brown fat tissue in newborn rodents (34). Finally, the beneficial effect of adequate PRL levels is highlighted by recent large cohort studies in men and women showing reduced levels of serum PRL in patients having glucose intolerance, T2D, and insulin resistance (35–37) and in children with metabolic syndrome and obesity (38), suggesting thereby that lower PRL levels contribute to disruption of metabolic homeostasis.

Here, we evaluated whether PRL promotes insulin sensitivity and adipose tissue metabolic health in obese rodents and whether in humans circulating and adipose tissue-produced PRL correlate with metabolic fitness markers systemically and in adipose tissue. We demonstrate that PRL administration counteracts obesity-induced insulin resistance and adipose tissue dysfunction in obese rats, whereas obese mice lacking PRL receptors show enhanced insulin resistance and unhealthy adipose tissue expansion. In agreement, PRL levels are reduced in insulin-resistant patients compared with insulin-sensitive ones and correlate directly with the levels of circulating adiponectin and with the expression of makers of fat fitness in both VAT and SAT. Our findings support the concept that lower concentrations of serum PRL in obesity have deleterious consequences for metabolic homeostasis and that increasing PRL levels may be a therapeutic aid against obesity-induced adipose tissue dysfunction and insulin resistance.

Materials and Methods

Experimental procedures in rats

Male Wistar rats bred for several years at our animal facility (the vivarium of the Institute of Neurobiology of the Universidad Nacional Autónoma de México [UNAM]) were housed at 22°C on a 12-hour light, 12-hour dark cycle with free access to food and water. Four-week-old rats were fed either a control diet (CD) (Laboratory rodent Diet 5001; LabDiet) or a high-fat diet (HFD) (Open Source Diet D12492; Research Diets) for 8 weeks. In the CD, 28%, 13.5%, and 58% of calories come from proteins, fat, and carbohydrates, respectively, whereas in the HFD, 20%, 60%, and 20% of calories come from proteins, fat, and carbohydrates, respectively. The fat source for both the CD and HFD is largely pig fat or lard. In the HFD, carbohydrates come from maltodextrin and sucrose and proteins from casein, whereas the CD contains oats, wheat, corn, soybean meal, wheat germ, cane molasses, fish meal, dried brewer's yeast, and porcine meat meal as sources of carbohydrate and protein. Thus, the CD is a balanced meal, whereas the HFD is high in fat and in simple sugars. After 4 weeks on the CD or HFD, rats were implanted sc or not with Alzet osmotic minipumps (DURECT Corp) releasing 0.16-mg/kg · d ovine PRL (Sigma-Aldrich) for 28 days. For this purpose, rats were anesthetized with ether inhaled for approximately 2 minutes, using an ether-soaked cotton ball placed at the bottom of a 50-mL conical tube (a new cotton was used for each animal and the cotton was never in direct contact with the animal), and the pumps were implanted sc in less than 2 minutes. To avoid stress-induced alterations in the amount of PRL released, animals were handled daily for 7 days before euthanization by CO_2 inhalation and decapitation. All studies were approved by the Bioethics Committee of the Institute of Neurobiology of the UNAM (protocol numbers 033 and 075), which complies with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (8th ed, National Academy Press).

Serum measurements

Rat PRL was evaluated by RIA. However, because the RIA for rat PRL does not detect ovine PRL, we used the Noble rat lymphoma (Nb2) assay to quantify the circulating levels of ovine PRL released by the osmotic minipump in the pump-implanted rats. This is a standardized method based on the proliferative response of Nb2 lymphoma cells to PRL (39). The Nb2 assay detects all PRLs (rat and ovine); therefore, we made this assay specific to ovine PRL by using antiovine PRL antibodies (National Hormone and Peptide Program; catalog number C-3581016789, Research Resource Identifier RRID AB_2629483) (see antibody in Table 1), that do not cross-react with rat PRL. Therefore, the difference in Nb2-bioactivity between the absence and presence of antiovine PRL quantified the levels of ovine PRL in rat serum. Rat adiponectin serum levels were measured using an ELISA kit (Millipore).

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Table 1. Antibody Table

Protein Target	Name of Antibody	Manufacturer, Catalog Number	Species Raised in; Monoclonal or Polyclonal	Dilution Used	RRID
Ovine PRL	Antiovine PRL-IC-1 antisera	National Hormone and Peptide Program, C-3581016789	Rabbit; polyclonal	1:1000	AB_2629483

Insulin tolerance test (ITT)

The ITT was performed in rats 3 weeks after delivering PRL via osmotic pumps (7 wk after the start of the CD or HFD). Glucose levels were measured with a glucometer in tail vein blood samples obtained with a lancet needle, before or 15, 30, 60, and 120 minutes after an ip injection of 0.75-U/kg insulin (Humulin R; Eli Lilly).

Adipose tissue histology

Subcutaneous inguinal and epididymal (visceral) adipose tissues from rats were fixed in 10% formalin, and sections of 10 μm were stained with hematoxylin and eosin. Adipocyte size was determined by calculating the mean adipocyte area from 9 fields per animal in 6–10 rats per group using the ImageJ software (https://www.imagej.nih.gov). The relative adipocyte number was calculated by dividing the fat pad weight by the mean adipocyte area in that fat depot.

Real Time-quantitative PCR (qPCR)

Adipose tissues were frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted using the TRIzol reagent (Invitrogen), and cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems). PCR products were detected and quantified in a Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories), using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) in a final reaction volume of 10 μL containing template and 0.5μM each of the primer pairs for rat (*Pparg, Il1b,* chemokine [C-C motif] ligand 2 [*Ccl2*], X-box-binding protein-1 [*Xbp1s*], and *Prlr*) genes. *18s rRNA* was used as housekeeping gene. Primer sequences are shown in Supplemental Table 1.

Statistics

Statistical data analysis was performed using SigmaStat 3.5 (Systat Software, Inc). Differences between groups were evaluated by two-way ANOVA followed by the Bonferroni's post hoc comparison test. Body weight and ITT curves were evaluated using repeated measures ANOVA. The threshold for significance was set at $P < .05$.

Experimental procedures in mice

C57BL/6 *Prlr*+/− mice were purchased from The Jackson Laboratory, and the colony was expanded and maintained for several generations before animals were used in our experiments. Animals were housed at 22°C on a 12-hour light, 12-hour dark cycle with free access to food and water. Eight- to 10-week-old male mice were fed a CD (Laboratory rodent Diet 5001; Lab-Diet) or a HFD (Open Source Diet D12492; Research Diets) for 8 weeks. To avoid stress-induced alterations in the amount of PRL released, animals were handled daily for 7 days before euthanization by CO_2 inhalation and decapitation. All studies were approved by the Bioethics Committee of the Institute of Neurobiology of the UNAM (protocol numbers 033 and 075), which complies with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (8th ed, National Academy Press).

Serum measurements

Mouse adiponectin serum levels were measured using an ELISA kit (Millipore).

ITT and glucose tolerance test (GTT)

ITT and GTT were performed in mice after 6 and 7 weeks, respectively, of CD or HFD. Glucose levels were measured with a glucometer in tail vein blood samples obtained with a lancet needle, before or 15, 30, 60, 90, and 120 minutes after an ip injection of 0.75-U/kg insulin (Humulin R; Eli Lilly) for the ITT, or 2 g/kg of 50% dextrose (PiSA Pharmaceuticals) for the GTT.

Adipose tissue histology

Subcutaneous inguinal and epididymal (visceral) adipose tissues from mice were fixed in 10% formalin, and sections of 7 μm were stained with hematoxylin and eosin. Adipocyte size was determined by calculating the mean adipocyte area from 9 fields per animal in 10–13 mice per group using the ImageJ software (https://www.imagej.nih.gov). The relative adipocyte number was calculated by dividing the fat pad weight by the mean adipocyte area in that fat depot.

qPCR

Adipose tissues were frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted using the TRIzol reagent (Invitrogen), and cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems). PCR products were detected and quantified in a Bio-Rad CFX96 RealTime System (Bio-Rad Laboratories), using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) in a final reaction volume of 10 μL containing template and 0.5μM each of the primer pairs for mouse (*Pparg, Il1b, Ccl2, Xbp1s,* and *Prlr*) genes. TATA box-binding protein (*Tbp*) was used as housekeeping gene. Primer sequences are shown in Supplemental Table 1.

Statistics

Statistical data analysis was performed using SigmaStat 3.5 (Systat Software, Inc). Differences between groups were evaluated by two-way ANOVA followed by the Bonferroni's post hoc comparison test. Body weight, ITT, and GTT curves were evaluated using repeated measures ANOVA. The threshold for significance was set at $P < .05$.

Experimental procedures involving human samples

Table 2 shows the clinical and biochemical characteristics of the subjects studied. Lean (BMI < 25), overweight (BMI = 25–29.9), and obese (BMI ≥ 30) mestizo male subjects, undergoing

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Table 2. Clinical and Biochemical Characteristics of the Subjects Studied

	Lean, BMI < 25 (n = 10)	Overweight, BMI = 25-29.9 (n = 11)	Obese, BMI > 30 (n = 6)	Overweight + Obese, BMI > 25 (n = 17)
Age (y)	35.9 ± 6.0	38.5 ± 2.9	38.8 ± 5.1	38.6 ± 2.5
BMI (kg/m^2)	22.5 ± 0.4	28.7 ± 0.4[a]	31.8 ± 0.5[a,b]	29.9 ± 0.5[a]
Insulin (pmol/L)	31.6 ± 6.0	65.2 ± 10[a]	65.1 ± 18	65.2 ± 9.0[a]
Glucose (mmol/L)	5.46 ± 0.4	6.50 ± 0.5	6.03 ± 0.7	6.34 ± 0.4
HOMA-IR	1.3 ± 0.3	3.17 ± 0.5[a]	2.79 ± 0.7	3.0 ± 0.4[a]
Adiponectin (μg/mL)	9.43 ± 1.4	6.39 ± 1.0	7.12 ± 1.5	6.6 ± 0.8
PRL (mg/L)	13.8 ± 1.2	11.5 ± 1.2	10.3 ± 2.5	11.1 ± 1.1

Values are mean ± SEM.

[a] $P < .05$ vs lean.

[b] $P < .05$ vs overweight.

surgery involving access to the abdominal cavity (appendectomy, abdominal hernia repair, or cholecystectomy), were recruited at the General Hospital of Queretaro between May 2012 and March 2014. The General Hospital internal review board and the Bioethics Committee of the Institute of Neurobiology, UNAM approved the study, and all procedures were performed in accord with the Declaration of Helsinki. Exclusion criteria were being younger or older than 18 or 65 years, respectively; having a medical history of liver or renal failure, and receiving medication with drugs known to affect PRL secretion (metoclopramide, methyldopa, opiates, or cimetidine). Fifty-four subjects were identified as candidates and signed the informed consent to participate. Serum samples were collected before surgery, and omental VAT and abdominal SAT samples were then obtained. Later, patients having PRL levels above basal values of 20 μg/L (n = 27) were excluded from the analysis, leaving 27 study subjects. Because PRL is a "stress hormone," we were concerned that values of PRL in these patients could be influenced by a stress-related confounding factor that could bias our analysis. The excluded patients were from both, insulin resistant (12 subjects), and insulin sensitive (15 subjects) groups.

Serum measurements

Human adiponectin serum levels were measured using ELISA kits (Millipore). Human PRL and insulin were measured by the Immulite 1000 Immunoassay Analyzer (Siemens), and glucose levels were evaluated by a colorimetric assay (Cayman Chemical).

qPCR

Adipose tissues were frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted using the TRIzol reagent (Invitrogen), and cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems). PCR products were detected and quantified in a Bio-Rad CFX96 RealTime System (Bio-Rad Laboratories), using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) in a final reaction volume of 10 μL containing template and 0.5μM each of the primer pairs for human (*ADIPOQ, PPARG, SLC2A4* [*GLUT4*], and *PRLR*) genes. *18S rRNA* was used as housekeeping gene. Primer sequences listed in Supplemental Table 1.

Statistics

Statistical data analysis was performed using SigmaStat 3.5 (Systat Software, Inc) and Microsoft Excel XLSTAT. Differences between 2 groups were evaluated by the Student's *t* test, and those between 3 or more groups by one-way ANOVA followed by the Bonferroni's post hoc comparison test. Spearman analysis for nonparametric data was used to evaluate correlations between serum levels of PRL and insulin, adiponectin, homeostasis model of insulin resistance (HOMA-IR), and gene expression levels in adipose tissue. The threshold for significance was set at $P < .05$.

Results

PRL treatment improves insulin sensitivity in diet-induced obese rats

Confirming our previous findings that circulating levels of PRL are reduced in obese rodents (40), after 8 weeks on a HFD, male rats showed lower serum PRL levels than animals fed a CD (Figure 1A).To explore whether reduced serum PRL levels contribute to the metabolic alterations seen in obesity, we increased systemic PRL with osmotic minipumps delivering the hormone during the last 4 weeks of the 8-week dietary intervention. PRL levels were increased to 4–6 times those in nontreated rats (Figure 1A). Although body weight and food consumption were unaltered by PRL treatment (Figure 1, B and C), elevating systemic PRL levels in HFD-fed animals improved metabolic responses, as evidenced by an enhanced insulin-induced, glucose-lowering effect during an ITT compared with animals on a HFD without PRL treatment (Figure 1D). In CD-fed animals, PRL treatment had no effect on insulin sensitivity (Figure 1D).

PRL prevents visceral adipocyte hypertrophy in obese rats

In the context of obesity, insulin sensitivity is attributed to a healthy expansion of the adipose tissue (1, 2, 41). This idea prompted us to evaluate whether PRL improves insulin sensitivity by promoting adipose tissue functionality. In HFD-fed animals, PRL treatment increased adipose tissue mass by 20% in the VAT and by 30% in the SAT depots beyond the increases induced by the HFD alone (Figure 2, A and D). This increase in adipose tissue mass

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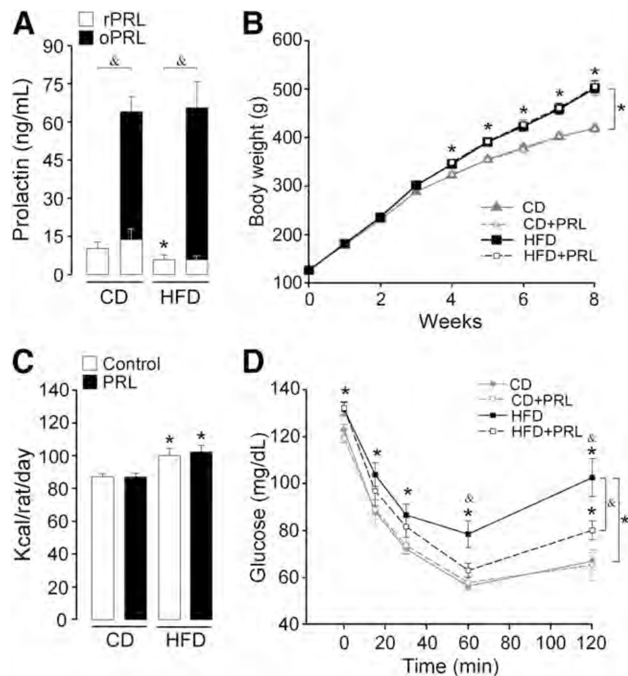

Figure 1. PRL treatment promotes insulin sensitivity in obese rats. Serum PRL levels after 8 weeks of CD or HFD in the absence or presence of osmotic minipumps delivering PRL during the last 4 weeks (n = 5–7) (A). White bars represent rat endogenous PRL (rPRL) measured by RIA; black bars represent ovine PRL (oPRL) from pumps quantified by Nb2 assay. Body weight (n = 7–14) (B), average food consumption (n = 6–7) (C), and ITT (n = 6–11) (D) at week 7 of the CD or HFD in rats treated or not with PRL. Data are mean ± SEM; *, $P < .05$ vs respective CD or CD+PRL; &, $P < .05$ vs respective diet without PRL. Statistically significant differences were determined with two-way ANOVA (A and C) or repeated measures ANOVA (B and D), followed by the Bonferroni's post hoc test.

was not reflected on body weight, because it represents only a 2% increase in total body mass. Compared with CD-fed animals, the number of adipocytes was greater in HFD-fed rats and was further elevated by PRL by 21% and 37% in SAT and VAT, respectively (Figure 2, B and E). Moreover, PRL treatment also reduced adipocyte hypertrophy in HFD-fed rats exclusively in VAT, whereas adipocytes in SAT of HFD-fed rats were the same size with and without PRL treatment (Figure 2, C and F). Under CD conditions, PRL treatment had no effect on adipose tissue mass, adipocyte number, or size (Figure 2, A-F). Because hypertrophic fat cells are believed to be metabolically dysfunctional and adipocyte hyperplasia is a healthier mechanism for increasing fat storage (2), these findings suggest that PRL can promote a healthier form of adipose tissue expansion under obesity conditions.

PRL increases *Pparg* and *Xbp1s* expression in visceral fat, reduces adipose tissue inflammation, and promotes adiponectin secretion

We next investigated the mechanisms that could mediate the beneficial actions of PRL on insulin sensitivity and adipose tissue expansion. In vivo, under HFD conditions PRL increased the expression of *Pparg* in the VAT but not in the SAT (Figure 2, G and H).

Increased inflammation is characteristic of fat tissue in obesity and has been shown to contribute to insulin resistance (42). PRL has been reported to exhibit both pro-and antiinflammatory actions in different cell types (43). PRL administration reduced by 50% the expression of *Il1b* in VAT from both CD- and HFD-fed animals (Figure 2G) and also reduced the expression of *Ccl2* in SAT from HFD-fed animals (Figure 2H).

The beneficial metabolic effects observed after elevating PRL levels in animals with HFD-induced obesity resemble those of adiponectin, an adipokine that is insulin sensitizing, proadipogenic, and antiinflammatory (44). Administration of PRL increased circulating adiponectin levels in rats on both the CD and HFD by 20% and 36%, respectively (Figure 2I). Recently, the transcription factor Xbp1 has been identified as a PRL target in the mammary gland fat pad (45), and the spliced form of Xbp1 has been shown to mediate increased adiponectin secretion by fat tissue in vivo (46). PRL treatment resulted in increased *Xbp1s* expression in the VAT of animals under both CD and HFD conditions (Figure 2J), implying that Xbp1s may contribute to PRL effects on adiponectin.

The preferential effect of PRL treatment on VAT in the rats (reducing adipocyte hypertrophy and increasing *Pparg* and *Xbp1s* expression) may be explained by the increased expression of PRL receptors in adipose cells from this depot (47). Interestingly, most of the effects of PRL on adipose tissue were observed under HFD feeding conditions. To explore whether the HFD could induce PRL receptor expression in fat thereby increasing PRL sensitivity, we evaluated the mRNA expression of the *Prlr* in the adipose tissue of rats under CD or HFD feeding conditions. We observed that *Prlr* expression is not changed by the HFD or by PRL treatment in either VAT or SAT (Supplemental Figure 1). Similar results were observed in adipose tissue from CD vs HFD-fed mice and from lean vs overweight/obese humans (Supplemental Figure 1).

PRL receptor-null mice under a HFD develop aggravated metabolic alterations

To further explore the role of PRL in metabolic health in obesity, *Prlr−/−* mice (on a C57BL/6 background) were fed a HFD for 8 weeks and studied for metabolic parameters. *Prlr−/−* mice gained more weight when fed a HFD (Figure 3A) and exhibited greater fat mass (Figure 3, B and C). This expansion of fat mass induced by the *Prlr* deletion was the result of enhanced adipocyte hypertrophy (Figure 3, D and E) in both fat depots with no significant change in adipocyte number per fat pad (Figure 3, F and G). *Prlr−/−* mice on a HFD showed exacerbated insulin

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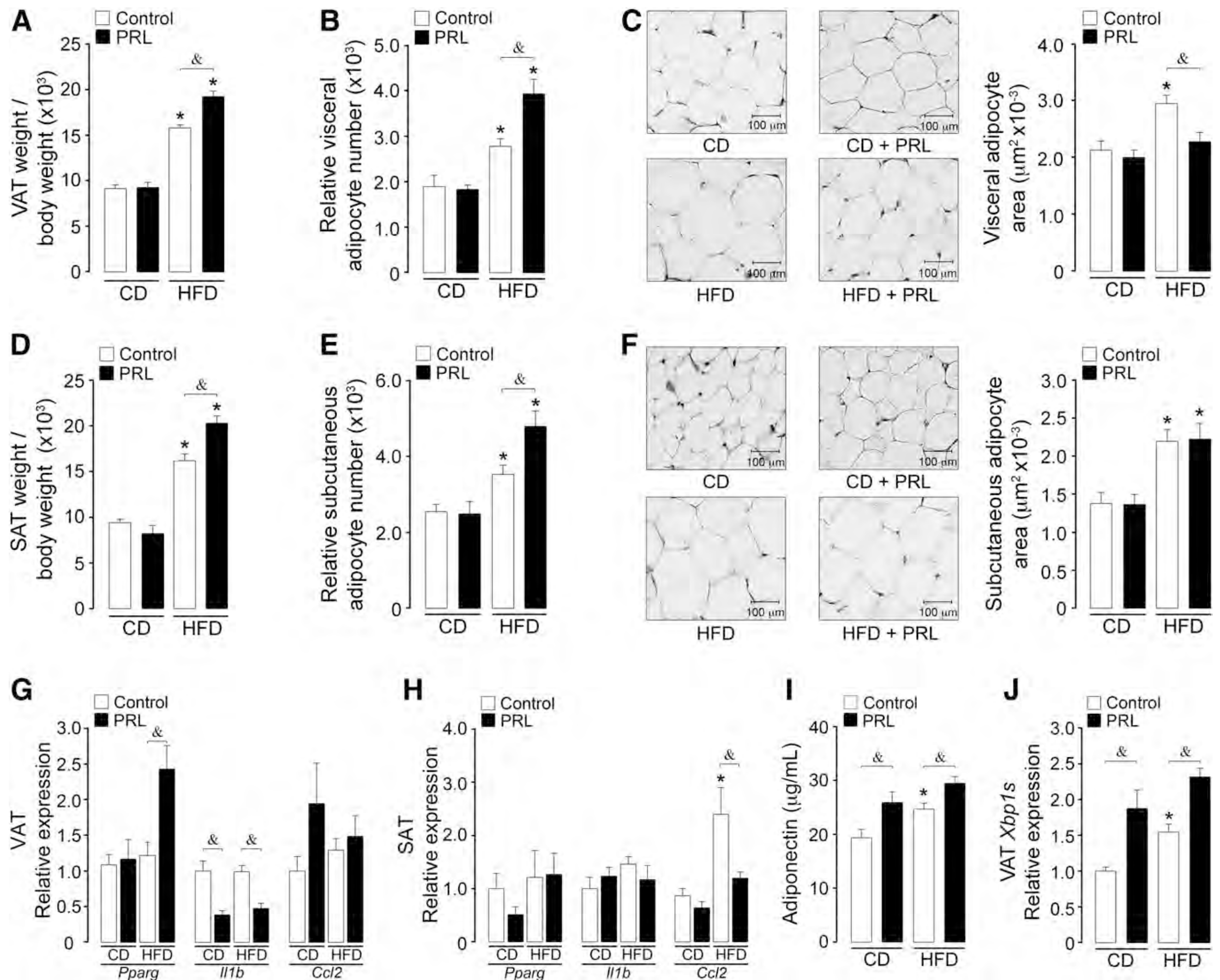

Figure 2. PRL treatment improves adipose tissue fitness in obese rats. VAT and SAT weight normalized to body weight (n = 7–13) (A and D), relative adipocyte number per fat depot (n = 6–10) (B and E), and representative sections of adipose tissue stained by hematoxylin and eosin to show adipocyte size (C and F). Graphs indicate quantification of adipocyte size in 9 fields per adipose tissue in 6–10 animals per group. Gene expression in VAT and SAT analyzed by qPCR (n = 7–13) (G and H). Serum adiponectin levels quantified by ELISA (n = 7–11) (I) and qPCR evaluation of *Xbp1s* in VAT (n = 5–6) (J). All evaluations were made after 8 weeks of the dietary intervention in the absence or presence of osmotic minipumps delivering PRL during the last 4 weeks. Data are mean ± SEM; *, $P < .05$ vs respective CD or CD+PRL; &, $P < .05$ vs respective diet without PRL. Statistically significant differences were determined with two-way ANOVA, followed by the Bonferroni's post hoc test.

resistance and glucose intolerance compared with wild-type mice (*Prlr+/+*) on the same diet (Figure 3, H and I). Inflammatory markers (*Il1b* and *Ccl2*) and serum adiponectin levels were not different between genotypes or diets in *Prlr−/−* vs *Prlr+/+* mice (Supplemental Figure 2).

PRL serum levels in patients associate positively with adiponectin levels and are reduced in insulin-resistant subjects

Establishing a correlation between PRL actions in rodent models and humans would support our hypothesis that circulating PRL plays a protective role against adipose tissue and metabolic dysfunction. To this end, we evaluated 27 lean, overweight, and obese male patients (characteristics in Table 2) and analyzed their circulating PRL levels, metabolic parameters, and markers of adipose tissue functionality. Lean, overweight, and obese groups did not differ in age, glucose, adiponectin or PRL levels. However, overweight and overweight plus obese groups had higher insulin levels and HOMA-IR values than the lean group (Table 2). In this population, serum PRL correlated inversely with serum insulin levels (Figure 4A) and HOMA-IR (Figure 4B) and positively with circulating adiponectin levels (Figure 4C). As expected, when the subjects were divided into insulin-sensitive and insulin-resistant groups (HOMA-IR cutoff = 3.0) (48), insulin-resistant subjects had a higher BMI (Table 3). Interestingly, the insulin-resistant subjects showed significantly ($P = .03$)

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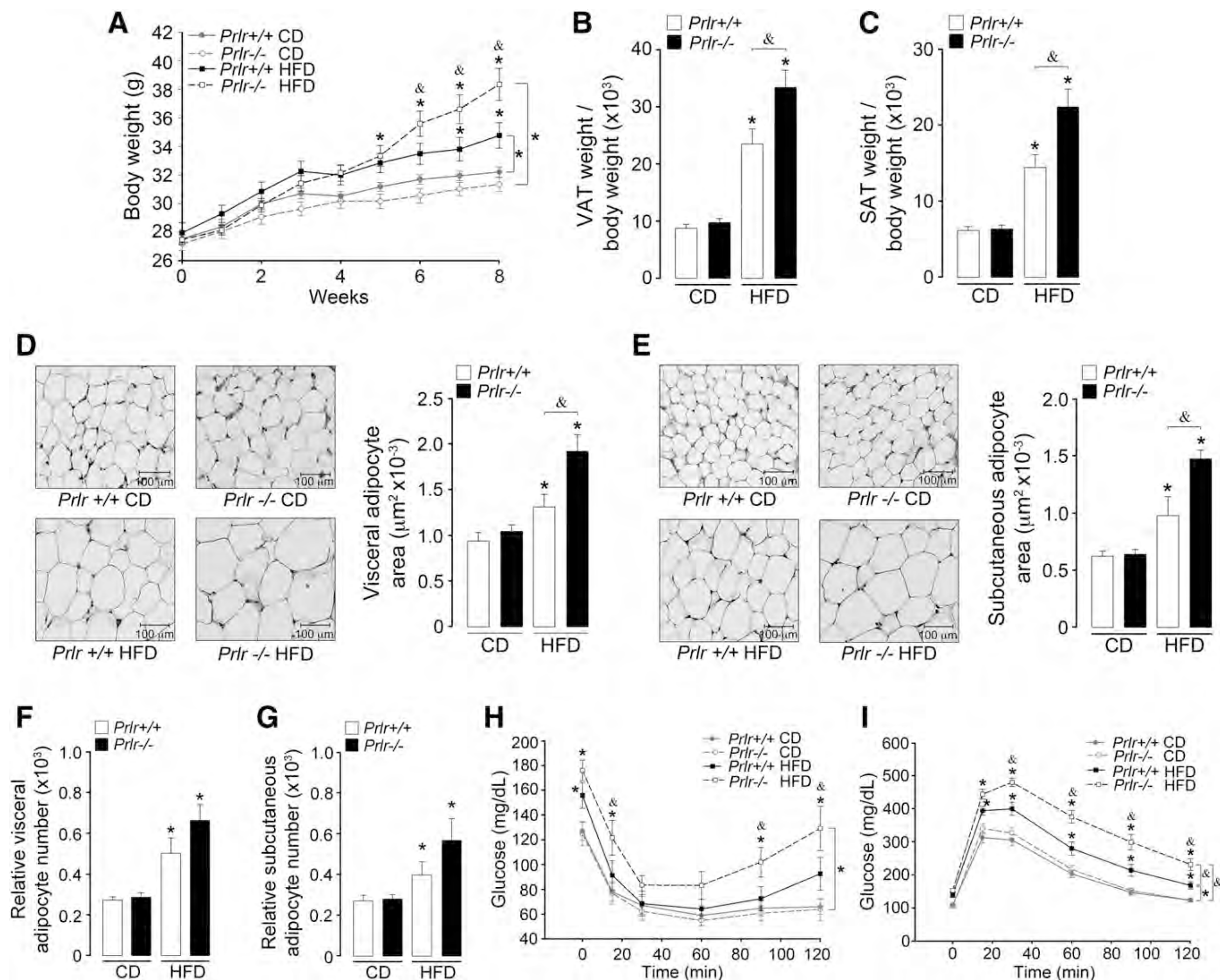

Figure 3. *Prlr−/−* mice fed HFD show aggravated insulin resistance and glucose intolerance and unhealthy adipose tissue expansion. *Prlr−/−* or *Prlr+/+* C57BL/6 mice were evaluated after 8 weeks of dietary intervention. Body weight (n = 26–29) (A), VAT and SAT weight normalized to body weight (n = 19–24) (B and C), representative sections of VAT and SAT stained by hematoxylin and eosin to show adipocyte size (D and E). Graphs indicate quantification of adipocyte size in 9 fields per adipose tissue in 10–13 animals per group. Relative adipocyte number per adipose depot (n = 10–13) (F and G). ITT after 7 weeks on CD or HFD (n = 11–13) (H). GTT after 6 weeks on CD or HFD (n = 24–29) (I). Data are mean ± SEM; *, $P < .05$ vs respective CD; &, $P < .05$ vs *Prlr+/+* on HFD. Statistically significant differences were determined with two-way ANOVA (B–G) or repeated measures ANOVA (A, H, and I), followed by the Bonferroni's post hoc test.

lower serum PRL levels than those who were insulin sensitive (Table 3). However, as mentioned before, when separated by BMI, overweight or obese groups did not show lower serum levels of PRL compared with the lean group (Table 2), indicating that reduced circulating PRL correlates with insulin resistance but not with obesity itself. Moreover, when subjects were divided by their circulating PRL levels, the higher PRL group showed reduced insulin resistance ($P = .005$) and increased adiponectin levels ($P = .004$) but similar BMI (Table 3).

PRL serum levels and PRL produced in adipose tissue positively associate with expression of adipose tissue fitness markers

The relationship between PRL and adipose tissue fitness was analyzed further by using VAT and SAT biopsies from lean, overweight, and obese patients (n = 15) undergoing abdominal surgery, and their circulating and adipose tissue PRL levels were evaluated for correlations with the expression of *PPARG, ADIPOQ,* and *GLUT4* in the fat tissue samples. Consistent with PRL promoting adipose tissue metabolic fitness, serum PRL levels correlated directly with the expression of *ADIPOQ* in both VAT and SAT, of *PPARG* in VAT, and of *GLUT4* in SAT (Figure 5, A and C). Moreover, PRL is produced by human adipose tissue, where it may act as an autocrine/paracrine factor (30). We found that *PRL* mRNA levels in adipose tissue correlated positively with the expression of *ADIPOQ* and *GLUT4* in SAT but not in VAT (Figure 5, B and D). These results suggest that both systemic PRL and PRL produced locally by the adipose tissue promote insulin sensitivity and adipose tissue metabolic homeostasis in humans.

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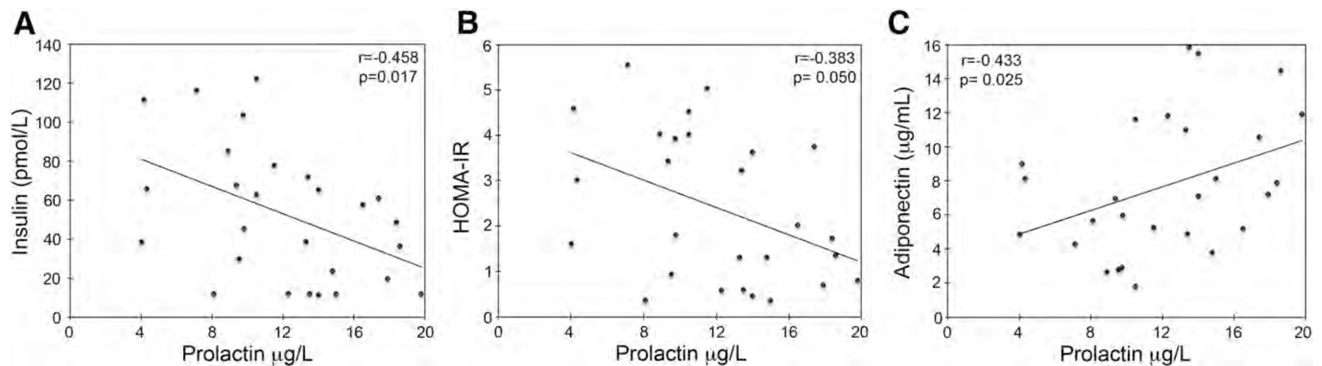

Figure 4. Serum PRL levels associate with insulin sensitivity and adiponectin levels in human subjects. Correlation analysis of serum PRL with circulating insulin (A), HOMA-IR (B), and adiponectin levels (C) from lean, overweight, and obese male subjects.

Discussion

During obesity, the inability of adipose tissue to maintain its functionality while storing nutrient surplus triggers insulin resistance and multiple metabolic diseases. Conversely, a healthy expansion of adipose tissue maintains and can even restore whole-body insulin sensitivity (41). Our study demonstrates that the hormone PRL promotes insulin sensitivity and the healthy expansion of adipose tissue and that these effects become apparent in both rodents and humans during obesity and insulin resistance, conditions that associate with low circulating PRL levels (35–38, 40). Mechanisms for the beneficial effects of PRL may include the ability to increase *Pparg* and *Xbp1s* expression and adiponectin secretion, as well as reducing adipocyte hypertrophy, increasing adipocyte hyperplasia, and reducing inflammation of adipose tissue. Also, other known effects of PRL may contribute to the overall beneficial effect of the hormone on metabolic homeostasis. In particular, PRL promotes β-cell function, not only in rodents but also in humans (27, 28), and stimulates insulin sensitivity in liver of mice, where overexpression of the *Prlr* promotes insulin sensitivity, whereas liver-specific deletion of the *Prlr* increases insulin resistance (29). Thus, together, these results imply that elevating the levels of PRL may have therapeutic value against obesity-induced metabolic dysfunction.

In obesity, excess fat is stored in existing adipocytes (hypertrophy) or in new fat cells (hyperplasia) generated from precursor cells by the process of adipogenesis (2, 3). Hyperplasia is generally regarded as a relatively benign and perhaps even protective mechanism of adipose tissue expansion (2, 3), whereas deficient adipogenesis and exaggerated adipocyte hypertrophy are linked to adipose tissue dysfunction and metabolic syndrome (1, 49). Here, we showed that PRL treatment stimulates adipocyte hyperplasia in VAT and SAT and reduces adipocyte hypertrophy in VAT of obese, insulin-resistant rats. Consistent with this observation, in the absence of PRL signaling *(Prlr−/−* mice), diet-induced obesity resulted in a greater level of adipocyte hypertrophy in both fat depots that is associated with increased insulin resistance and glucose intolerance. The beneficial effects of PRL likely involve a direct action on adipocytes. PRL receptors are expressed in fat (50, 51), both in adipocyte precursor cells and mature adipocytes (47), and their levels are higher in visceral than in sc fat cells (47). In addition, *Prlr* expression increases in the 3T3-L1 cell line during differentiation to adipocytes, and

Table 3. Insulin-Resistant Subjects Show Reduced PRL Serum Levels

Insulin Sensitivity	Sensitive (HOMA-IR < 3.0), (n = 15)	Resistant (HOMA-IR > 3.0), (n = 12)	*P*
Age (y)	37.4 ± 4.0	38.1 ± 3.4	.893
BMI (kg/m^2)	25.4 ± 1.0	29.3 ± 0.9	.008
HOMA-IR	1.1 ± 0.1	4.1 ± 0.2	<.000001
Adiponectin (μg/mL)	8.8 ± 1.2	6.3 ± 0.9	.091
PRL (μg/L)	13.7 ± 1.1	10.1 ± 1.1	.031
PRL Levels	**Higher (12–20 μg/L) (n = 14)**	**Lower (4–11.9 μg/L) (n = 13)**	***P***
Age (y)	37.5 ± 4.1	37.9 ± 3.0	.938
BMI (kg/m^2)	26.3 ± 1.0	28.1 ± 1.2	.251
HOMA-IR	1.6 ± 0.3	3.3 ± 0.5	.005
Adiponectin (μg/mL)	9.7 ± 1.1	5.5 ± 0.8	.004
PRL (μg/L)	15.6 ± 0.6	8.3 ± 0.7	<.000001

Values are mean ± SEM.

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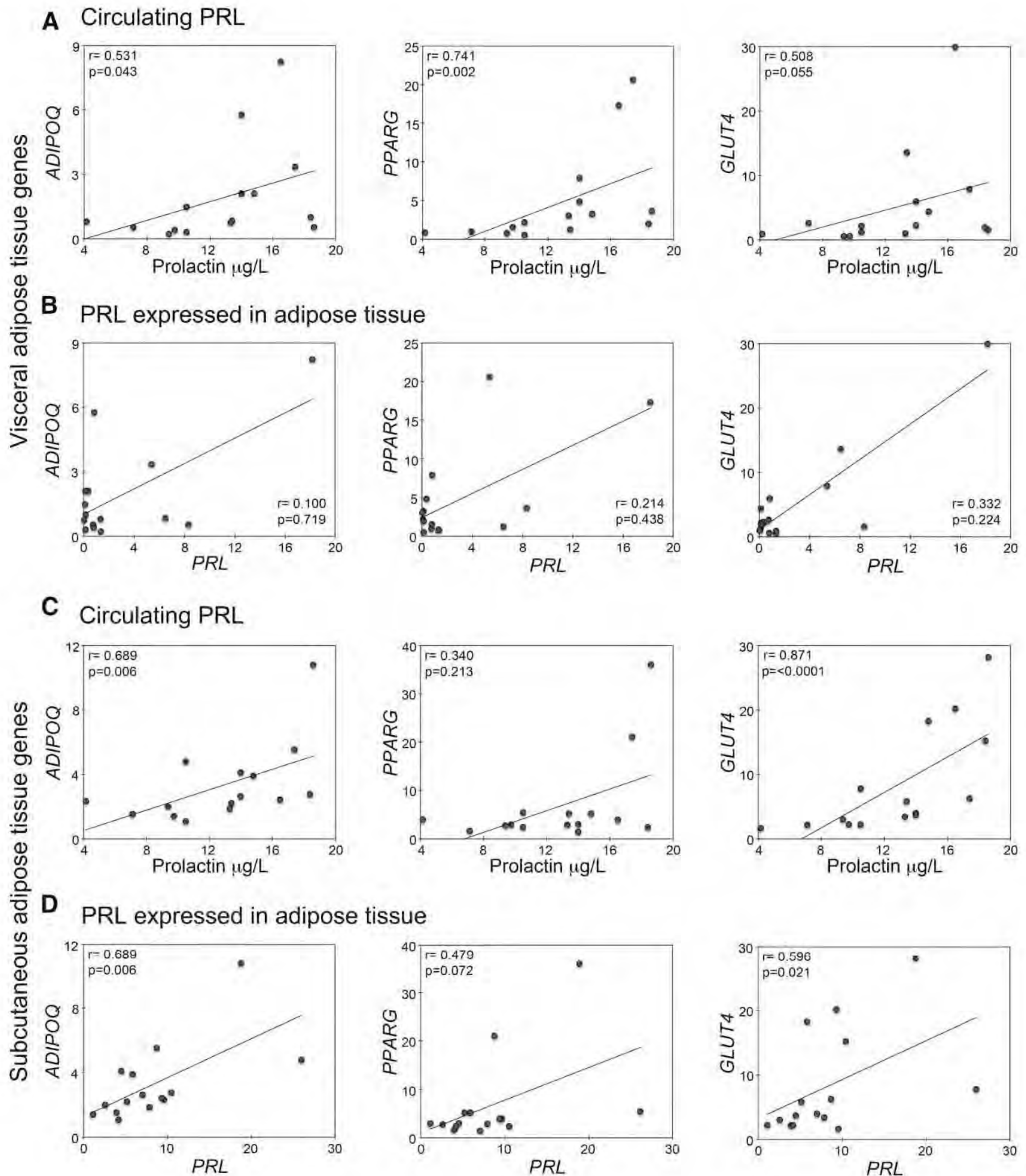

Figure 5. Serum PRL and adipose tissue *PRL* mRNA levels associate with the expression of adipose tissue fitness markers. Correlation analysis of (A and C) serum PRL or (B and D) adipose tissue *PRL* mRNA, and mRNA expression levels of adipose tissue fitness markers in VAT (A and B) and SAT (C and D).

PRL acts on these cells to stimulate such differentiation (52). These effects occur through activation of Signal transducer and activator of transcription 5 (52), as well as increased expression of *Cebpb* and *Pparg* (33), which promotes adipogenesis.

Here, we show that PRL promotes *Pparg* expression only in VAT and exclusively under HFD conditions. In view of the known ability of PPARg to improve insulin sensitivity (53), it is likely to mediate the PRL protective effects. PPARg induces the expression

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of adiponectin (54), an adipokine promoting insulin sensitivity in rodents and humans (55). We found that elevating PRL in serum increases circulating adiponectin levels in rats under both control and HFD conditions. However, PRL stimulated *Pparg* expression only under HFD conditions, so the increase in adiponectin levels induced by PRL under CD likely involves other factors. One such factor may be Xbp1s, a transcription factor activated by PRL in mammary adipose tissue during lactation (45). We found that PRL up-regulates *Xbp1s* in the VAT of rats under both CD and HFD feeding, Xbp1s induces adipogenesis via the transcriptional activation of *Cebpa* and *Pparg* (56, 57) and improves glucose tolerance and insulin sensitivity in lean and obese mice by increasing adiponectin secretion (46).

It is puzzling that PRL treatment increases *Xbpl* levels in adipose tissue under both CD and HFD but increases *Pparg* only under HFD. Several studies have shown that many PPARg effects occur in association with obesity or HFD conditions (58–61). One mechanistic explanation for this is that visceral fat (but not SAT) from obese mice is more sensitive to PPARg agonists (rosiglitazone) (59). Enhanced sensitivity results from increased expression of ubiquitin hydrolase/ligase ubiquitin C-terminal esterase L1 in visceral fat of obese mice and humans. Ubiquitin hydrolase/ligase ubiquitin C-terminal esterase L1 facilitates the proteasomal degradation of the PPARg heterodimerization partner retinoid X receptor a (RXRa), promoting increased PPARg-RXRb heterodimerization, which is less sensitive to transcriptional repression by silencing mediator of retinoid and thyroid hormone receptors than PPARg-RXRa. Thus, the reduced RXRa to RXRb ratio increases PPARg transcriptional activity (59), thereby promoting *Cebpa* expression and initiating the positive feedback loop between these 2 factors to mutually increase each other's expression. Accordingly, by favoring the action of PPARg agonists or activators, the obese condition provides a plausible explanation for the preferential effects of PRL treatment under HFD.

In our study, increasing serum PRL to levels (60–80 ng/mL) equivalent to those reported in male rats at circadian peaks (62) or in response to stress (63, 64) elevated systemic adiponectin. This implies that PRL at a relatively high concentration but within a physiological range found in female reproductive states may protect against metabolic dysfunction by increasing circulating adiponectin. However, contrary to this proposal, PRL reduces adiponectin secretion by adipose tissue in culture (65) and transgenic female (but not male) mice overexpressing PRL and having PRL serum levels (>250 ng/mL, at the high end of the normal range found during pregnancy and lactation), show lower systemic adiponectin values (65). Although the differences in in vitro vs in vivo conditions, gender, physiological state, and PRL concentration may contribute to these discrepancies, clinical work supports our proposal. Clinical studies demonstrate that circulating PRL positively correlates with insulin sensitivity (36, 38) and with serum adiponectin in males (current findings) and in females with polycystic ovary syndrome (66).

The inhibition of proinflammatory mediators is another way by which PRL may improve insulin sensitivity and fat fitness. In agreement with the antiinflammatory effects of PRL in other rodent models of disease (67), we found that PRL treatment reduces the expression of *Il1b* and *Ccl2* in VAT and SAT, respectively, and both IL-1b and CCL2 contribute to inflammation-mediated insulin resistance and adipose tissue dysfunction in obesity (68, 69).

Consistent with our observations in *Prlr*−/− mice, mice deficient in PRL (on a C57BL/6 strain) fed a CD have normal adipose tissue mass and unaltered metabolic phenotype (70). Although insulin sensitivity and adipose tissue fitness have not been evaluated in PRL null mice under a HFD, we show here that *Prlr*−/− mice on a HFD develop increased obesity, exacerbated glucose intolerance, insulin resistance, and enlarged adipocytes. These findings confirm the beneficial actions of PRL on metabolic and adipose tissue function and show that its effects become evident during obesity. However, in apparent contradiction to our findings, *Prlr*−/− mice on a 129/SvJ genetic background display milder obesity and no insulin resistance in response to a HFD (71), which prompts to the emergence of beige/brite adipocytes in white fat. The 129/SvJ strain is intrinsically less prone to diabetes and HFD-induced obesity than the C57BL/6 strain (72). Its reduced susceptibility involves increased intermuscular deposits of brown adipocytes (72), higher thermogenic capacity, augmented beiging potential, and distinct inflammatory pathways in the adipose tissue and gut microbiota environments (73, 74). The aggravated metabolic alterations found in the present study in *Prlr*−/− mice of the C57BL/6 strain were consistent with the fact that no beige adipocytes could be detected in SAT or VAT under HFD conditions (Figure 3, D and E).

The above information makes clear that in rodents, as in humans, metabolic alterations depend on genetically associated risk and protective factors and that these factors are able to influence the outcome of PRL metabolic effects. However, our conclusion that the absence of PRL action in obesity has negative metabolic consequences that can be counteracted by increasing PRL levels, is supported by clinical data. Low PRL

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levels associate with glucose intolerance, insulin resistance, obesity, and T2D in adults (35–37) and with metabolic syndrome in children (38). Here, we extend these findings by showing that insulin-sensitive male subjects have PRL values higher than those in insulin-resistant patients, regardless of their BMI (within the 19–36 range), and that patients' serum PRL levels associate positively with circulating adiponectin levels and with the expression of adipose tissue fitness markers, including *ADIPOQ, PPARG,* and *GLUT4*. Furthermore, our work supports the role of PRL as a local regulator of human adipose tissue (30) by showing that PRL synthesis in SAT correlates with the expression of *ADIPOQ* and *GLUT4* in this adipose depot.

In summary, the present study shows that PRL protects against obesity-induced insulin resistance by promoting the healthy expansion and metabolic fitness of adipose tissue via mechanisms that include increased levels of *Xbp1s, Pparg,* and adiponectin in fat. Given that low circulating PRL correlates with disrupted metabolic homeostasis in patients, restoring or elevating PRL levels may help reduce metabolic dysfunction in normal or obese individuals with insulin resistance. Our work expands the knowledge of PRL effects in physiological states beyond pregnancy and lactation, and highlights the need for a better understanding of its role in regulating metabolic homeostasis in both females and males.

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Prolactin anterior pituitary expression and circulating levels are reduced in obese and diabetic rats: role of TGF-β and TNF-α

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Lemini M, Ruiz-Herrera X, Ledesma-Colunga MG, Díaz-Lezama N, De los Ríos EA, López-Barrera F, Méndez I, Martínez de la Escalera G, Macotela Y, Clapp C. Prolactin anterior pituitary expression and circulating levels are reduced in obese and diabetic rats: role of TGF-β and TNF-α. *Am J Physiol Regul Integr Comp Physiol* 308: R792–R799, 2015. First published February 25, 2015; doi:10.1152/ajpregu.00327.2014.—The levels of the hormone prolactin (PRL) are reduced in the circulation of patients with Type 2 diabetes and in obese children, and lower systemic PRL levels correlate with an increased prevalence of diabetes and a higher risk of metabolic syndrome. The secretion of anterior pituitary (AP) PRL in metabolic diseases may be influenced by the interplay between transforming growth factor β (TGF-β) and tumor necrosis factor α (TNF-α), which inhibit and can stimulate AP PRL synthesis, respectively, and are known contributors to insulin resistance and metabolic complications. Here, we show that TGF-β and TNF-α antagonize the effect of each other on the expression and release of PRL by the GH4C1 lactotrope cell line. The levels of AP mRNA and circulating PRL decrease in high-fat diet-induced obese rats in parallel with increased and reduced AP levels of TGF-β and TNF-α mRNA, respectively. Likewise, AP expression and circulating levels of PRL are reduced in streptozotocin-induced diabetic rats and are associated with higher AP expression and protein levels of TGF-β and TNF-α. The opposing effects of the two cytokines on cultured AP cells, together with their altered expression in the AP of obese and diabetic rats suggest they are linked to the reduced PRL production and secretion characteristics of metabolic diseases.

prolactin; TGF-β; TNF-α; obesity; diabetes

OBESITY AND ITS ASSOCIATED disorders, such as Type 2 diabetes, constitute the most prominent socioeconomic health-related burden in the developed world and parts of the underdeveloped world. Obesity and diabetes are characterized mainly by alterations in glucose and lipid metabolism but also in the hormonal and cytokine milieu. Understanding how such changes arise and lead to further complications is essential to developing effective therapies against these metabolic diseases. Prolactin (PRL) is a peptide hormone secreted mainly by the anterior pituitary gland (AP) and is known for its fundamental role in lactation; however, PRL exerts a wide variety of actions in reproduction, osmoregulation, brain function, immune response, and angiogenesis (16, 25). A very relevant, but still underappreciated, role of PRL is the regulation of energy homeostasis and, particularly, its possible contribution to the development and progression of metabolic syndrome and diabetes. Recently, large cohort studies have shown that high circulating PRL levels correlate with reduced prevalence of glucose intolerance and Type 2 diabetes in adults (6, 60) and that low PRL serum levels are observed in obese children compared with lean controls and constitute a predictive factor for progression toward metabolic syndrome (15). Therefore, identifying factors responsible for the downregulation of PRL-circulating levels in obesity and diabetes has potential value for the treatment of these metabolic disorders.

In the present work, we investigated whether the production of PRL is downregulated in the AP of obese and diabetic rats and evaluated whether alterations in the AP expression of TGF-β and/or TNF-α correlated with these changes. TGF-β and TNF-α increase in the circulation of obese and diabetic patients (15, 19, 29, 61, 63) and play important roles in insulin resistance and diabetes pathophysiology (59, 61). Also, these cytokines and their receptors are ubiquitously expressed in a variety of tissues from rodents and humans, including the AP PRL-producing cell (lactotrope) (17, 65), where TGF-β inhibits (24, 49) and TNF-α can stimulate (27, 36), but also inhibit (30, 58), PRL synthesis and release; however, there is little or no information regarding the actions of TNF-α or TGF-β on PRL secretion in the context of metabolic diseases.

MATERIALS AND METHODS

GH4C1 cell culture. GH4C1 cells are a subclone of the cell line GH3 isolated from an AP rat tumor that produces both growth hormone and PRL (56). The stock line was maintained between passages 3 and 10. GH4C1 cells were cultured in F10 medium (Sigma Chemicals, St. Louis, MO) supplemented with 15% heat-inactivated horse serum (Gibco, Invitrogen, Carlsbad, CA), 2.5% heat-inactivated FBS (Gibco), and 1% penicillin-streptomycin at 37°C in 5% CO_2. Subconfluent GH4C1 cell cultures (5×10^5 cells/well in 24-well plates or 10^6 cells/well in 12-well plates) were plated and incubated for 48 h. Medium was then changed to high-glucose DMEM-containing 0.5% FBS and antibiotics for 24 h, and the cells were treated with different concentrations of TGF-β and/or TNF-α for 48 h. Recombinant human TGF-β and TNF-α were purchased from R&D Systems (Minneapolis, MN). The concentrations of both cytokines were comparable to those used previously in similar in vitro studies (18, 27). At the end of the incubation, the cells were counted, and their viability was evaluated by the dye-exclusion method.

Animal models of obesity and diabetes. Male Wistar rats were housed under standard laboratory conditions (22°C; 12:12-h light-dark cycle, free access to food and water). All animal procedures were approved by the Bioethics Committee of the Institute of Neurobiology of the National University of Mexico and comply with the U.S. National Research Council's *Guide for the Care and Use of Laboratory Animals* (8th ed., National Academy Press, Washington, D.C.). To induce obesity, 4-wk-old rats were fed with a 60% high-fat diet

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(HFD) (Open Source Diet D12492; Research Diets) or a standard chow (CD) in which 13% of calories comes from lipids (Laboratory rodent Diet 5001, LabDiet, Richmond, IN). To induce diabetes, male Wistar rats (250–300 g) were injected with a single intraperitoneal dose of streptozotocin (STZ; 60 mg/kg in citrate buffer, at pH 4.5) (Sigma-Aldrich, St. Louis, MO) or vehicle (citrate buffer) after an overnight fast. Rats with a blood glucose concentration ≥250 mg/dl were considered diabetic. After 10 wk on the HFD or 6 wk after the STZ injection, animals were anesthetized by CO_2 inhalation followed by decapitation between 1000 and 1400 to determine the AP mRNA and circulating levels of PRL, TGF-β, and TNF-α. TGF-β and TNF-α protein levels were also evaluated in the AP of control and diabetic rats 6 wk after STZ injection. To avoid stress-induced PRL release, animals were handled daily for 7 days before death.

Blood glucose and insulin tolerance test. After rats were either fasted overnight or not fasted, blood glucose levels were measured with a glucometer. Blood was collected from the tip of the tail using a lancet needle. ITT was performed by intraperitoneal injection of 0.75 U/kg insulin (Humulin R, Eli Lilly, Indianapolis, IN) after a 1-h fast, and blood glucose was evaluated before (*time 0*) and at 15, 30, 60, and 120 min after insulin.

Real-time quantitative RT-PCR. Total RNA was extracted from frozen AP or cultured GH4C1 cells using the guanidine isothiocyanate method, and cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Warrington UK). PCR products were detected and quantified with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Auburn, AL) in a 10-μl final reaction volume containing template and 0.5 μM of each of the primer pairs for rat *PRL*: forward 5′-TGG CAG AAC AGA AGG TTT GA-3′, reverse 5′-CCA TGA ACA GCC AAG TGT CA-3′; rat *TNF-α*: forward 5′-GGG CTT GTC ACT CGA GTT TT-3′, reverse 5′-TGC CTC AGC CTC TTC TCA TT-3′; and rat *TGF-β*: forward 5′-CAC GAT CAT GTT GGA CAA CTG CTCC-3′, reverse 5′-CTT CAG CTC CAC AGA GAA GAA CTGC-3′. Amplification performed in the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA) included a denaturation step of 10 min at 95°C, followed by 40 cycles of amplification (10 s at 95°C, 30 s at the primer pair-specific annealing temperature and 30 s at 72°C). The PCR data were analyzed by the $2^{-\Delta\Delta CT}$ method, and cycle thresholds were normalized to the housekeeping genes *GAPDH* (in AP) or *18S* (in GH4C1 cells) to calculate the expression levels of genes of interest. Different housekeeping genes were used because the expression of GAPDH was very stable in the APs from the different groups of rats, but not in GH4C1 cells, where its expression varied among treatments. 18S was a good constitutive expression gene in the cell line.

PRL, TGF-β, and TNF-α levels. PRL was measured in serum of CD- and HFD-fed rats by conventional radioimmunoassay (RIA) using standard procedures and reagents provided by the National Hormone and Pituitary Program and by Dr. A. F. Parlow (Harbor University of California, Los Angeles Medical Center) or in control and STZ-induced diabetic rats by the Nb2 cell bioassay, a standard procedure based on the proliferative response of the Nb2 lymphoma cells to PRL (55). Serum TGF-β and TNF-α were quantified by ELISA from R&D Systems (Minneapolis, MN) and BD Biosciences (San Diego, CA), respectively. AP TGF-β and TNF-α levels were measured by ELISAs from Cloud-Clone (Houston, TX) and Sigma-Aldrich (St. Louis, MO), respectively, that were designed to evaluate tissue lysates.

Statistics. All data were replicated in three or more experiments. The statistical analyses were performed using the Sigma Stat 7.0 (Sigma Stat 7.0; Systat Software, San Jose, CA) and the GraphPad Prism (GraphPad Software, La Jolla, CA) software. Statistical differences were determined by the Student's *t*-test (between two groups) or by ANOVA (for more than two groups). Differences comparing a combination of two factors (TGF-β or TNF-α) were analyzed by a two-way ANOVA and Bonferroni's post hoc test. The effect of different doses of only TGF-β were evaluated using one-way ANOVA followed by Bonferroni's post hoc test. The threshold for significance was set at 5%.

RESULTS

Opposing effects of TGF-β and TNF-α on PRL expression and release by GH4C1 cells. Consistent with the effects shown on AP primary cultures (36, 49), TGF-β inhibits (18, 24), while TNF-α stimulates (27), PRL expression by the GH3 or GH4 lactotrope cell lines. Here, we confirm these findings in GH4C1 cells, a cell line derived from the somatomammotropic GH3 cells, which in contrast to their precursors primarily produce PRL (56). We show that treatment with different concentrations of TGF-β reduced the levels of PRL mRNA in cell lysates (Fig. 1, *A* and *B*) and of PRL in the cell-conditioned media (Fig. 1, *E* and *F*). Both effects were prevented and, in some cases, turned into stimulation by the coaddition of a high concentration of TNF-α (50 ng/ml) (Fig. 1, *B* and *F*); moreover, stimulation by this TNF-α concentration was reduced in the presence of the higher concentrations of TGF-β (Fig. 1, *B* and *F*). Similarly, we found that TNF-α, at various concentrations, stimulates PRL mRNA and secreted protein levels, and showed that stimulation was blocked by the coincubation with a maximal inhibitory concentration of TGF-β (10 ng/ml) (Fig. 1, *C* and *G*). The antagonism between the two factors was further illustrated when cells were treated with high concentrations of both cytokines simultaneously, in which case, the levels of PRL mRNA and secreted protein were significantly different from those observed when the cells were treated with each cytokine alone and similar to those of the control, untreated cells, essentially cancelling each other's effects (Fig. 1, *D* and *H*). Neither TGF-β nor TNF-α at their high inhibitory (10 ng/ml) or high stimulatory (50 ng/ml) concentration modified the viability or the number of cells (Fig. 1, *I* and *J*).

PRL, TGF-β, and TNF-α AP mRNA and serum levels are modified in HFD-fed rats. Next, we investigated whether PRL levels were altered in rats fed a HFD, a widely accepted model of obesity and metabolic syndrome (1). After 10 wk on a HFD, the rats showed a 30% increase in body weight (Fig. 2*A*), and they were hyperglycemic in fasted and fed states (Fig. 2*B*) and insulin-resistant, as evaluated by the insulin tolerance test (Fig. 2*C*). While both groups responded similarly to a high bolus of exogenous insulin, glucose levels remained higher at all time points in HFD-fed rats compared with controls. Similar curves are commonly observed in this type of experiment and imply that, because of insulin resistance, endogenous insulin is not able to maintain normoglycemia.

The HFD-fed rats showed a 46% reduction in AP PRL mRNA levels, in line with a similar decrease (45%) in serum PRL levels (Fig. 3, *A* and *D*). TGF-β mRNA values increased by 3.5-fold, and those of TNF-α decreased by 56% in the AP of HFD-fed rats compared with CD-fed animals (Fig. 3, *B* and *C*). The levels of both TGF-β (P = 0.008) and TNF-α (P = 0.006) increased in the circulation of obese animals (Fig. 3, *E* and *F*).

PRL, TGF-β, and TNF-α AP mRNA and serum levels are modified in STZ-induced diabetic rats. We then investigated whether similarly altered PRL, TGF-β, and TNF-α AP mRNA and serum levels found in the HFD-induced obesity model could be observed in other diabetes models, particularly in the STZ-treated rat, a well-characterized model of Type 1 diabetes

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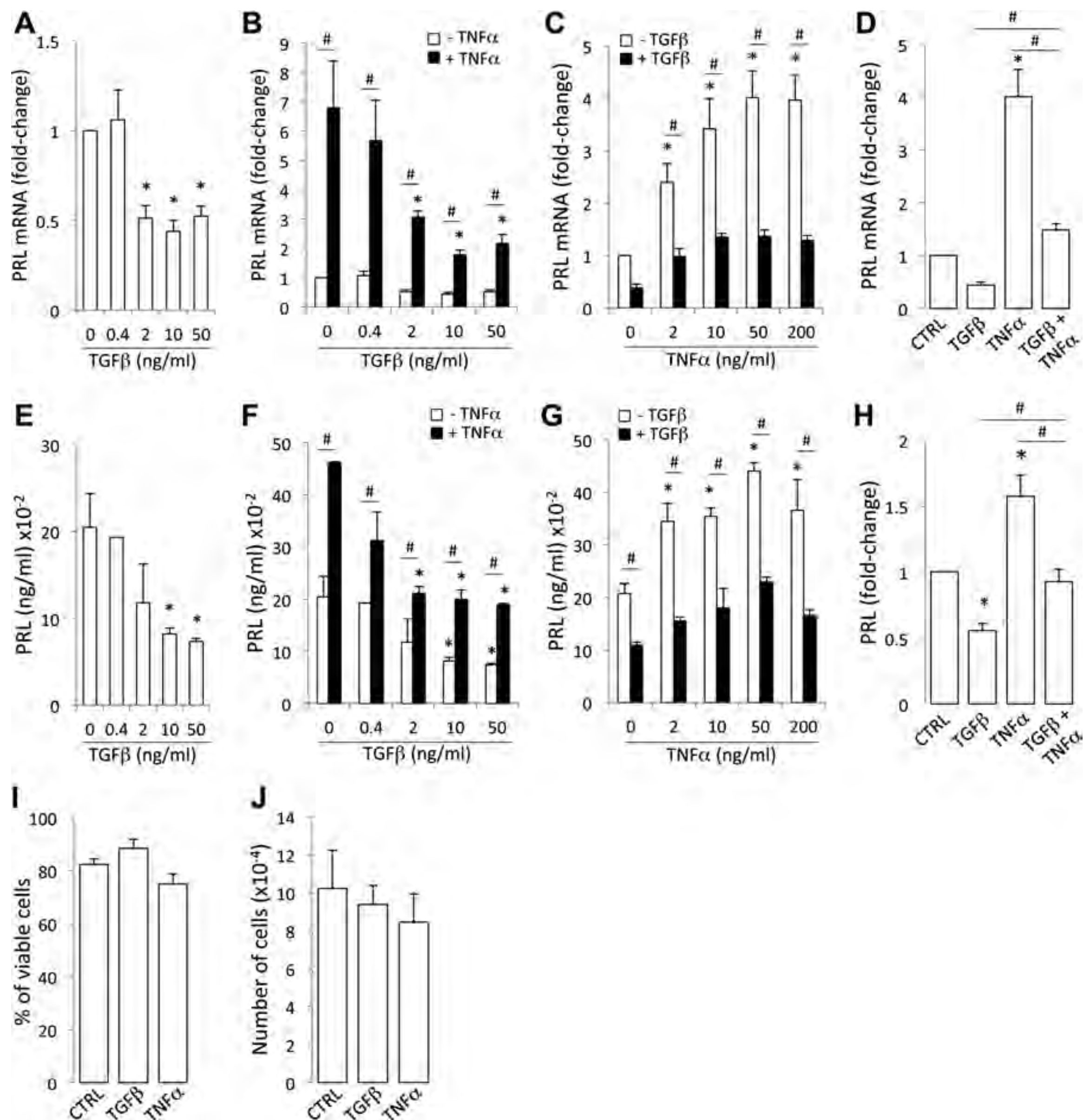

Fig. 1. Antagonism of TNF-α and TGF-β actions on prolactin (PRL) expression and release in GH4C1 cells. PRL mRNA expression was evaluated by qRT-PCR in cell lysates (*A, B, C, D*), and PRL levels were measured by RIA in the culture medium (*E, F, G, H*) of GH4C1 cells treated or not with a series of TGF-β (*A, B, E, F*) or TNF-α (*C, G*) concentrations with (*B, F*) or without high TNF-α (50 ng/ml) (*A, B, E, F*) or TGF-β (10 ng/ml) (*C, G*). PRL expression (*D*) and protein levels are shown in culture medium (*H*) and in the absence (CTRL) or presence of 10 ng/ml TGF-β or 50 ng/ml TNF-α alone or in combination. Viability relative to total number of cells (*I*) and number of cells after incubation (*J*) with or without TGF-β (10 ng/ml) or TNF-α (50 ng/ml) during 48 h. Values are expressed as means ± SE from four independent experiments. *$P < 0.05$ vs. 0, analyzed by a one-way ANOVA and Bonferroni comparison test (*A, E*). *$P < 0.05$ vs. respective 0 or CTRL. #$P < 0.05$ vs. each cytokine alone, analyzed by two-way ANOVA and Bonferroni comparison test (*B, C, D, F, G, H*).

(33). Six weeks after a single intraperitoneal injection of STZ, rats showed a 21% reduction in body weight and marked hyperglycemia, compared with control, vehicle-treated rats (Fig. 4, *A* and *B*). PRL AP mRNA and circulating levels were reduced by 26% and 85%, respectively, in STZ-treated compared with control rats (Fig. 5, *A* and *D*). Of note, the serum PRL values in control rats were slightly higher than expected for normoprolactinemia and may reflect a mild stress response to the handling procedure. AP TGF-β and TNF-α mRNA values increased significantly in STZ-injected rats ($P = 0.0006$ and $P = 0.03$ vs. control for TGF-β and TNF-α, respectively) (Fig. 5, *B* and *C*). Moreover, in line with their mRNA levels, the protein values of both cytokines increase in the AP of STZ-treated rats compared with control animals ($P = 0.048$ and $P = 0.002$ vs. control for TGF-β and TNF-α, respectively) (Fig. 5, *B* and *C*). The serum levels of TGF-β were reduced ($P = 0.011$) in STZ-treated rats compared with control rats, while those of TNF-α were increased ($P = 0.047$) (Fig. 5, *E* and *F*).

DISCUSSION

PRL functions as a metabolic hormone. It affects metabolic homeostasis by promoting the proliferation, survival, and in-

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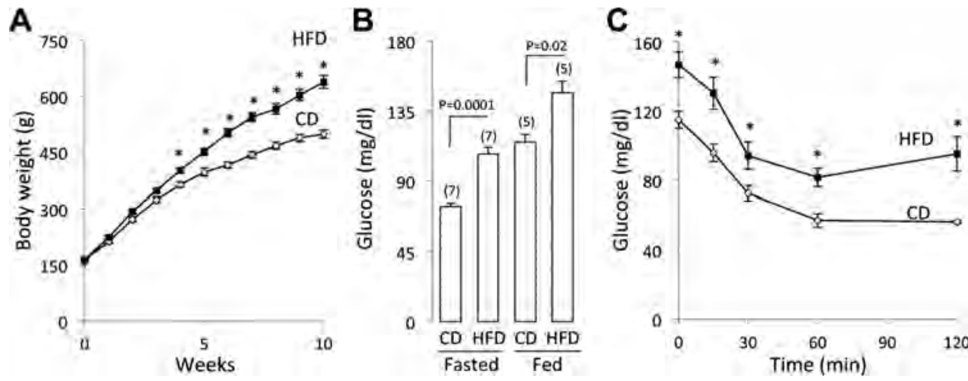

Fig. 2. High-fat diet for 10 wk induces increased body weight, hyperglycemia, and insulin resistance in rats. Body weight (*A*), blood glucose levels in the fed and fasted states (*B*), and insulin tolerance (*C*) were evaluated in rats fed with a control (CD) or with a high-fat diet (HFD) for 10 wk. Values are expressed as means ± SE of 5–12 animals per group. *$P < 0.05$ vs. CD analyzed by Student's *t*-test.

sulin production of pancreatic β-cells (26, 57), and by stimulating food intake (11), liver insulin sensitivity (64), and adipose tissue growth and function (8). Large cohort clinical studies showed that reduced PRL serum levels, within the physiological range, are associated with diabetes and impaired glucose regulation (6, 60). Also, lower PRL circulating levels in obese children correlate with increased insulin resistance, inflammation, and body mass index (15). Consistent with the clinical studies, circulating levels of PRL are reduced in STZ-treated diabetic rats (9, 54), in mice rendered obese by chemical means (51), and in the *ob/ob* (38) and *db/db* mouse (50) models of diabetes and obesity. The 24-h pattern of plasma PRL levels is also disrupted in HFD-fed male rats (13), and serum PRL levels are reduced in HFD-fed female mice (52).

Here, we confirmed the reduced circulating levels of PRL found in STZ-induced diabetes in rats and found lower PRL serum levels in obese male rats fed a HFD. Moreover, we show that PRL mRNA levels are also reduced in the AP of both STZ-induced diabetic and HFD-fed obese rats, suggesting that downregulation of PRL synthesis contributes to the decrease in systemic PRL. Lower PRL mRNA levels may also reflect a reduction in the number of lactotropes. Along this line, the AP PRL content and the number of AP PRL secretory granules and lactotropes are reduced, and lactotrope apoptosis is increased in STZ-induced diabetic rats (3, 4, 62).

Dopamine (DA) is the main inhibitor of AP PRL synthesis and release (7), and several DA-dependent and independent factors may act centrally and locally to regulate AP PRL synthesis and release in metabolic diseases (37, 43). Increased fat mass, hyperinsulinemia, and hyperleptinemia may modify PRL secretion in obesity and diabetes (37). However, both HFD-fed obese rats and STZ-induced diabetic rats show reduced AP PRL synthesis and release despite their opposite changes in weight gain (Refs. 1 and 33, present results), leptin (5, 53), and insulin (33, 44) levels, suggesting that other factors

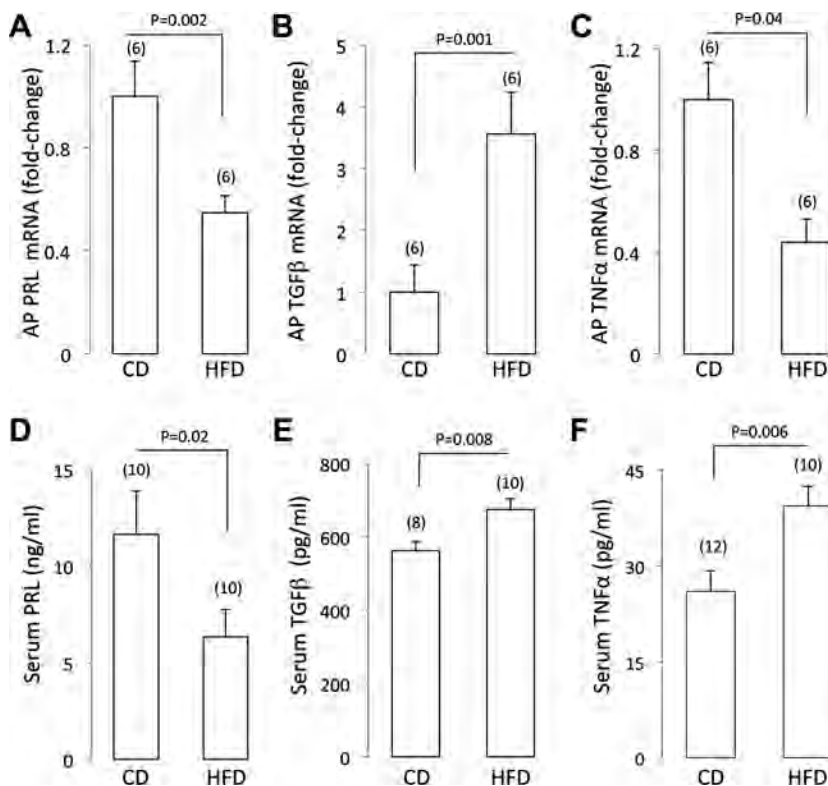

Fig. 3. PRL, TGF-β, and TNF-α anterior pituitary expression and their serum levels are altered in HFD-induced obese rats. Anterior pituitary (AP) expression of PRL (*A*), TGF-β (*B*), or TNF-α (*C*) were evaluated by qRT-PCR, serum levels of PRL (*D*) were evaluated by RIA, and those of TGF-β (*E*) and TNF-α (*F*) by ELISA in rats fed with control (CD) or with a high-fat diet (HFD) for 10 wk. Values are expressed as means ± SE. Numbers inside parentheses indicate *n* values. *P* values are provided above each panel.

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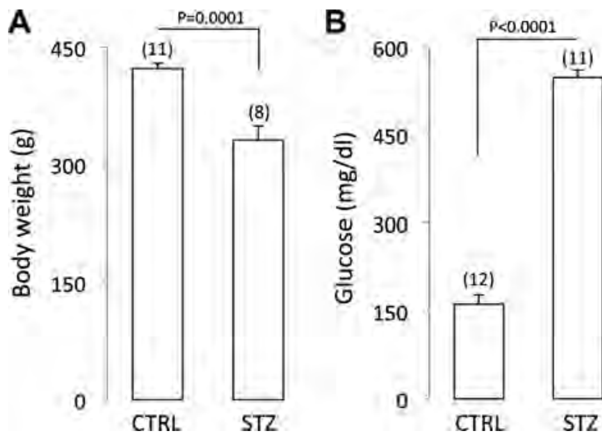

Fig. 4. Streptozotocin injection results in body weight loss and hyperglycemia in rats. Body weight (*A*) and blood glucose levels (*B*) were evaluated in rats 6 wk after a single intraperitoneal injection of vehicle (CTRL) or streptozotocin (STZ; 60 mg/kg). Values are expressed as means ± SE. Numbers inside parentheses indicate *n* values. *P* values are provided above each panel.

besides increased fat mass, hyperleptinemia, and hyperinsulinemia may affect PRL levels in these animals. In search of PRL regulators common to both models, we evaluated TGF-β and TNF-α.

TGF-β and TNF-α increase in the circulation of obese and diabetic humans (15, 19, 29, 61, 63), HFD-fed rodents (20, 61), and STZ-diabetic rats (4, 21, 22). TGF-β promotes adiposity and glomerular pathology in obesity and diabetes, and both the adipose and renal tissues are major sources of TGF-β released into the circulation (14, 22, 23, 61). Also, TNF-α is produced and released by stressed adipocytes from fat depots and by activated macrophages from tissues such as fat, muscle, and liver to have local and systemic effects on metabolism, inflammation, and vascular function (29, 31). Of note, both cytokines have direct effects on PRL secretion. TGF-β inhibits and TNF-α can stimulate PRL synthesis and release by cultured lactotropes (18, 24, 27, 36, 49). Because both cytokines increase in obesity and diabetes, we investigated the outcome of their combined treatment on PRL secretion by the GH4C1 lactotrope cell line. We observed that TGF-β inhibited and TNF-α stimulated PRL synthesis and release in a dose-dependent manner, and these effects did not appear to involve altered cell viability or proliferation. Notably, the two cytokines antagonized the effect of each other, suggesting that their relative concentrations could determine whether PRL synthesis is upregulated or downregulated. We hypothesized that, if these cytokines were involved in the PRL downregulation seen in obesity and diabetes, TGF-β inhibitory effects would prevail over the stimulatory action of TNF-α.

Our findings measuring the circulating concentrations of both cytokines agree with studies showing increased TGF-β levels in HFD-fed rodents and of TNF-α in HFD-fed and STZ-treated rats. However, in contrast to previous work (22), TGF-β systemic levels were reduced in STZ-treated diabetic rats. The reason for this discrepancy is unclear. TGF-β in serum may represent TGF-β released by the kidney due to diabetic nephropathy (14). It is possible that the earlier stage of nephropathy associated with the shorter exposure to diabetes in our study (6 vs. 10 wk) influenced TGF-β circulating levels. However, TGF-β and TNF-α values affecting PRL secretion in the pituitary milieu may not necessarily mirror their circulating levels. Both cytokines are expressed in the AP. The production of TGF-β appears to be restricted to folliculostellate cells (32) and lactotropes (10). In the latter, TGF-β synthesis is upregu-

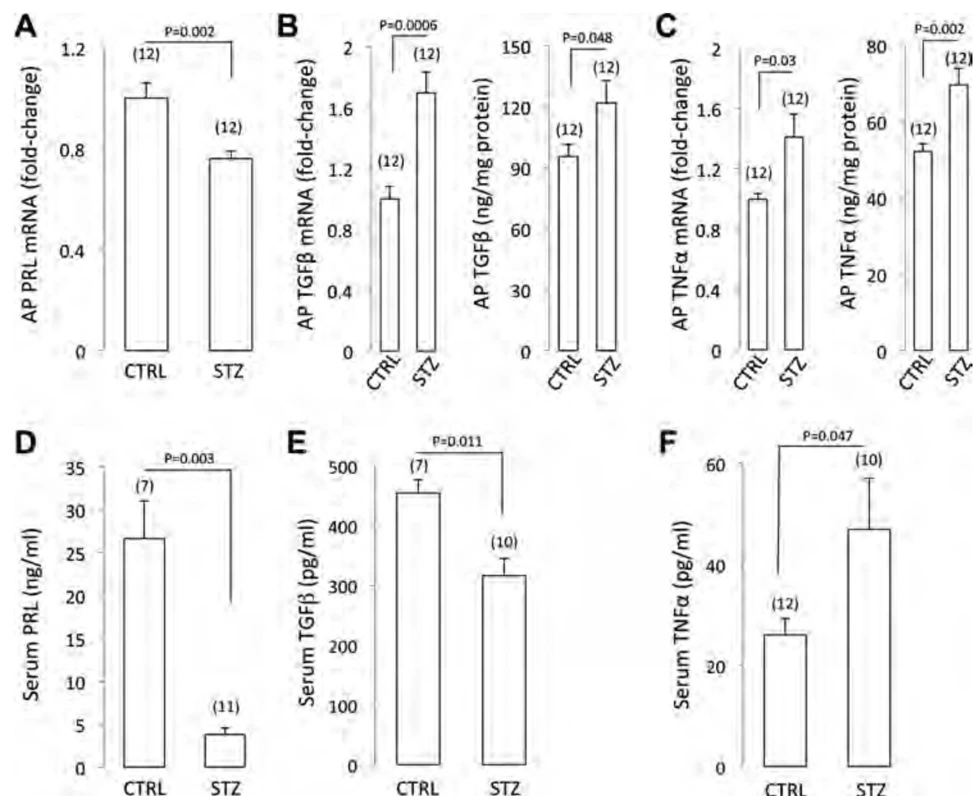

Fig. 5. Levels of PRL, TGF-β, and TNF-α mRNA in the anterior pituitary and the corresponding proteins in serum are altered in STZ-induced diabetic rats. Anterior pituitary (AP) mRNA levels of PRL (*A*), TGF-β (*B*), and TNF-α (*C*) were evaluated by qRT-PCR, serum levels of PRL (*D*) were evaluated by the Nb2 cell bioassay, and AP and serum levels of TGF-β (*B*, *E*) and TNF-α (*C*, *F*) were determined by ELISA in rats 6 wk after a single intraperitoneal injection with vehicle (CTRL) or STZ. Values are expressed as means ± SE. Numbers inside parentheses indicate *n* values. *P* values are provided above each panel.

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lated by dopamine and downregulated by estrogens to modulate PRL secretion and lactotrope proliferation (10, 46, 48). TNF-α is produced by AP macrophages and somatotrophs (2). Therefore, an important question is whether the levels of TGF-β and TNF-α are altered in the AP under the conditions of obesity and diabetes.

To our knowledge, this is the first report showing that the expression of TGF-β increases and that of TNF-α decreases in the AP of HFD-induced obese rats, reciprocal changes that, if translated into opposite protein levels, could together explain the decrease in PRL synthesis and release. We also show that the AP mRNA and protein levels of TGF-β and TNF-α are upregulated in STZ-induced diabetic rats. The increase in TNF-α expression is consistent with a previous report showing elevated levels of the TNF-α protein in the AP of STZ-induced diabetic rats that were investigated as a mechanism inducing the apoptosis-mediated loss of lactotropes occurring in diabetes (4).

The higher expression of both TGF-β and TNF-α in the AP of diabetic rats, the reduced levels of circulating TGF-β in diabetes, and the systemic rise of TNF-α in obesity and diabetes are difficult to reconcile with the lower expression and circulating levels of PRL. From the cell line data, an increase in TNF-α would cancel out the inhibitory effect of TGF-β on PRL. A possible explanation for a prevailing effect of TGF-β in spite of the upregulation of TNF-α may relate to the endogenous concentrations of the two cytokines and their receptor binding affinities. In diabetic rats, the AP concentrations are similar (122 vs. 72 pg/mg of protein for TGF-β and TNF-α, respectively) but the K_d value (50 pM) of the TGF-β receptor-2 (TGF-β R-2) is 20- and 7-fold lower than the K_d values of the TNF-α R-1 (1.23 nM) and the TNF-α R-2 (0.35 nM), respectively (40, 47). TGF-β R2 forms a heteromeric complex with TGF-β R1 and is essential for all TGF-β-induced signaling (40, 45). Moreover, the circulating levels of TGF-β measured in diabetic (316 pg/ml, i.e., 25.3 pM) and in HFD-fed (674 pg/ml, i.e., 53.9 pM) rats are similar to the K_d concentration of the TGF-β R-2, whereas the systemic levels of TNF-α in diabetic (47 pg/ml, i.e., 2.8 pM) and HFD-fed (39 pg/ml, i.e., 2.3 pM) rats are more than 400- and 100-fold lower than the K_d values of the TNF-α R-1 and TNF-α R-2, respectively. Therefore, it is possible that TGF-β would be more effective than TNF-α at their endogenous levels. Alternatively, it should also be noted that the effect of TNF-α may not occur or may turn into inhibition, depending on complex in vivo interactions. Concentrations of TNF-α similar to those found to be stimulatory in our study, may have either no effect (41), inhibit PRL release (30, 58), or promote lactotrope apoptosis (12), depending on the duration of TNF-α exposure, and on the sex, influence of gonadal steroids, and age of animals from which the cells were obtained. Also, lactotrope apoptosis occurs in STZ-treated rats (4), so it is possible that STZ-mediated upregulation of TNF-α could help lower AP PRL indirectly by promoting lactotrope apoptosis.

The altered pituitary TGF-β and TNF-α expression profiles and circulating levels found in obesity and diabetes, together with the direct reciprocal effects of the two cytokines on AP PRL synthesis and release, suggest that TGF-β and TNF-α play both local and systemic regulatory roles to inhibit PRL secretion in metabolic diseases. Evaluating whether specific, in vivo blockage of the two cytokines in the AP milieu modifies PRL secretion should test this putative causative link.

The functional interaction between TGF-β and TNF-α raises the question of what controls their AP production in obesity and diabetes. TGF-β is upregulated by DA in lactotropes (46, 48) and may be linked to the overactivation of hypothalamic DA occurring in obesity (51, 52) and diabetes (54). Production of TNF-α in the AP could be a stress response (34). TNF-α can affect the secretion of ACTH by AP cells (28), and TNF-α is upregulated in the AP by a relatively weak, systemic inflammatory stress (34), which occurs in obesity (39) and Type 1 diabetes (42). Chronic poor metabolic control also triggers TNF-α expression (21), and hypoglycemia stimulates PRL secretion in poorly controlled diabetic patients (35). Further research is needed to investigate whether the altered expression of TGF-β and TNF-α in the AP could influence metabolic disorders by other mechanisms besides PRL.

Perspectives and Significance

The present study demonstrates that reduced AP PRL secretion in obese and diabetic rats correlates with altered changes in the AP expression of two metabolically relevant cytokines, TGF-β and TNF-α. We hypothesize that an imbalance in the AP between the two cytokines, derived from their altered local and systemic levels, favors PRL downregulation in metabolic disorders. These findings help establish the context of TGF-β and TNF-α regulation of PRL, a hormone with recognized influence on metabolic homeostasis that, when downregulated, may aggravate metabolic alterations resulting from obesity and diabetes. However, further studies are needed to prove these hypotheses.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.L., Y.M. and C.C. conceived and designed research; M.L., X.R-H., M.G.L-C., N. D-L, F.L-B. and I.M. performed experiments; M.L., E.A.D.l.R., Y.M., and C.C. analyzed data; M.L. G.M.d.l.E., Y.M. and C.C interpreted results of experiments; M.L. and Y.M. prepared figures; Y.M. and C.C. wrote the manuscript; M.L., G.M.d.l.E. edited and revised manuscript; M.L., X.R-H., M.G.L-C., N. D-L., E.A.D.l.R., F.L-B., I.M., G.M.d.l.E., Y.M. and C.C. approved final version of manuscript.

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1 PROLACTIN REGULATES LIVER GROWTH DURING POSTNATAL
2 DEVELOPMENT IN MICE
3
4
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23 **ABSTRACT**

24 The liver grows during the early postnatal period first at slower and then at
25 faster rates than the body to achieve the adult liver-to-body weight ratio (LBW), a
26 constant reflecting liver health. The hormone prolactin (PRL) stimulates adult liver
27 growth and regeneration and its levels are high in the circulation of newborn
28 infants, but whether PRL plays a role on neonatal liver growth is unknown. Here,
29 we show that the liver produces PRL and upregulates the PRL receptor in mice
30 during the first 2 weeks after birth, when liver growth lags behind body growth. At
31 postnatal week 4, the production of PRL by the liver ceases coinciding with the
32 elevation of circulating PRL and the faster liver growth that catches up with body
33 growth. PRL receptor null mice (*Prlr-/-*) show a significant decrease in the LBW at
34 1, 4, 6, and 10 postnatal weeks and reduced liver expression of proliferation (cyclin
35 D1, *Ccnd1*) and angiogenesis (platelet/endothelial cell adhesion molecule 1,
36 *Pecam1*) markers relative to *Prlr+/+* mice. However, the LBW increases in *Prlr-/-*
37 mice at postnatal week 2 concurring with the enhanced liver expression of *Igf-1*
38 and the liver upregulation and downregulation of suppressor of cytokine signaling 2
39 (*Socs2*) and *Socs3*, respectively. These findings indicate that PRL acts locally and
40 systemically to restrict and stimulate postnatal liver growth. PRL inhibits liver and
41 body growth by attenuating growth hormone-induced *Igf-1* liver expression via
42 *Socs2* and *Socs3*-related mechanisms.

43

44 **Keywords**: postnatal liver growth; prolactin; growth hormone; Igf-1; suppressor of
45 cytokine signaling-2 and 3

46

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47 **INTRODUCTION**

48 The liver is relatively immature at birth in rodents and humans and
49 undergoes dynamic changes in growth and differentiation during early postnatal life
50 to develop its functional capacity (19). During the first 2 postnatal weeks (PNW),
51 the growth of the rodent liver lags behind body growth and the fraction of body
52 weight contributed by the liver at birth is not recovered until the PNW 4, when the
53 liver-to-body weight ratio (LBW) reaches adult levels (13, 39). While some factors
54 promoting postnatal liver growth have been deciphered (2, 26, 44), those restricting
55 liver growth during the first 2 weeks of life are basically unknown. Negative and
56 positive factors involved in growth of the newborn liver may be similar to those
57 causing the cessation of adult liver growth, the upregulation of liver size during
58 female reproductive states, such as pregnancy (11) and lactation (24), and the
59 termination of liver regeneration once the correct liver size has been reached (33).
60 Understanding these factors could help manage liver diseases such as cancer and
61 cirrhosis. Prolactin (PRL), the hormone essential for lactation, may be one such
62 factor.

63 PRL stimulates adult liver growth and regeneration by promoting hepatocyte
64 proliferation and angiogenesis (34, 37). The circulating levels of PRL are high
65 under conditions of physiological liver growth such as in pregnant and lactating
66 females (4), in rodents after partial hepatectomy (7), and in the neonate. PRL is
67 markedly elevated (>100 ng/ml) in human newborns during the first 5 weeks of life
68 (21) and in rats around PNW 4 (38, 45). However, the physiological function of
69 PRL in the neonate is unknown. The present study examines the role of PRL on
70 liver growth at birth and during PNW 1 to 10 in mice null (*Prlr-/-*) or not (*Prlr+/+*) for

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71 the PRL receptor (PRLR). We report that the liver produces PRL during the first 2
72 PNW concurring with enhanced liver expression of the long *Prlr* isoform and
73 undetectable PRL circulating levels. Moreover, PRL promotes liver growth during
74 postnatal development, except at PNW 2 when it inhibits liver and body growth and
75 interferes with growth hormone (GH)-signaling mechanisms. This inhibition may
76 help explain the insensitivity to GH occurring in rodent liver at this time, which
77 characterizes the GH-independent phase of body growth.
78

79 **MATERIALS AND METHODS**

80 *Animals*. C57BL/6 *Prlr+/-* mice from The Jackson Laboratory were colony
81 expanded and maintained for several generations in our vivarium. Nulliparous
82 C57BL/6 *Prlr+/-* pregnant mice were allowed to give birth, and *Prlr+/+* and *Prlr-/-*
83 pups were anesthetized by CO_2 inhalation and euthanized by decapitation at birth
84 or at different PNW. Sexual dimorphism occurs in the liver and has been largely
85 attributed to sex differences in circulating GH profiles and GH-signaling leading to
86 male related pubertal corporal growth (16, 49). However, these and other sex-
87 related changes do not occur in prepubertal animals (9, 30). Because our results in
88 liver and body growth were similar throughout PNW 1 to 6, data from male and
89 female pups were compiled and analyzed without regard to sex. All animals were
90 housed under standard laboratory conditions and treated according to local
91 institutional guidelines and in compliance with the *Guide for the Care and Use of*
92 *Laboratory Animals* published by the U.S. National Institutes of Health. The
93 Bioethics Committee of the Institute of Neurobiology of the National University of
94 Mexico (UNAM) approved all animal experiments.

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95 *Liver weight and liver-to-body weight ratio (LBW).* Liver was dissected out,
96 blotted free of blood on filter paper, and weighed. The LBW was calculated as the
97 weight of the organ divided by the body weight of the animal at sacrifice.
98 *ELISA***.** Serum and liver PRL levels were measured using the ultrasensitive
99 ELISA (20) with a lower limit of detection of 0.2 ng/ml and an intra-assay coefficient
100 of variation of 8.1%.
101 *Quantitative RT-PCR.* Total RNA was extracted from liver tissue using
102 TRIzol reagent (Invitrogen, Carlsbad, CA, USA), cDNA was synthesized using the
103 High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City,
104 CA, USA), and quantitative PCR was performed using the Maxima SYBR
105 Green/ROX qPCR Master Mix (Fermentas, Hanover, MD, USA) with the CFX96
106 PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Primers were
107 as follows: *Prlr*: 5'-ACACGCGCAGATCTCCTTACCA-3' and 5'-
108 CCCCTTGCACAGCCACTT-3'; *Prl:* 5'-AACCTGATCCTCAGTTTGGTG-3' and 5'-
109 CCTCAATCTCTTTGGCTCTTG-3'; cyclin D1 (*Ccnd1)*: 5´-
110 ACTGGTCCCCTGAGGTCTGAGT-3´ and 5´-ACATGATCCTCCAAACCTCTTCTC-
111 3´; *Vegf*: 5´-CGCGAGTCTGTGTTTTTGCA-3´ and 5´-
112 CAGAGCGGAGAAAGCATTTGT-3´; platelet/endothelial cell adhesion molecule 1
113 (*Pecam1):* 5´-AGCAGGACAGGTCCAACAAC-3´ and 5´-
114 CAAGCAAAGCAGTGAAGCTG-3´; GH receptor (*Ghr):* 5'-
115 CCAACTCGCCTCTACACC-3´ and 5'-GGGAAAGGACTACACCACCTG-3'; *Ifg-1:*
116 5'-CTGAGCTGGTGGATGCTCTT-3' and 5'-CACTCATCCACAATGCCTGT-3';
117 suppressor of cytokine signaling 3 (*Socs3*): 5'-
118 SOCS3CCCGCGGGCACCTTTCTTAT-3´ and 5'-

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119 CACTGGATGCGTAGGTTCTTGGTC-3'; *Socs2*: 5'-
120 TGTGAGTCCCAACCTAGTGC-3' and 5'-GTAGAAGGGAGGCAGCTGTT-3';
121 cyclophilin A (*CypA)*: 5'-GGCGGCAGGTCCATCTACG-3´ and 5'-
122 CTTGCCATCCAGCCATTCAGTC-3´; and *Gapdh*: 5'-AACGACCCCTTCATTGAC-
123 3´ and 5'-TCCACGACATACTCAGCAC-3'. Results are expressed as arbitrary units
124 after normalization to *Gapdh* and *CypA* as housekeeping genes.

125 *Statistics*. Analysis was performed by one-way ANOVA followed by the
126 Tukey's test using the Sigma Stat 7.0 software (Systat Software, San Jose, CA).
127 The threshold for significance was set at P<0.05.

128

129 **RESULTS**

130 *Liver and body growth during postnatal development***.** The liver and body
131 weights increased during the early postnatal period and stabilized in young adults
132 at PNW 8 (Figure 1A, B). As previously observed (13, 39), the liver weight fell
133 slightly behind that of the body during the first 2 PNW and increased sharply
134 thereafter. Therefore, the percentage of total body weight accounted by the liver
135 (LBW) fell throughout the first 2 PNW and then rose to adult levels at PNW 4
136 (Figure 1C). These results indicate the presence of mechanisms able to initially
137 restrict and then accelerate neonatal liver growth.

138 *The liver is under the influence of PRL throughout postnatal development***.**
139 The adult liver is the organ with highest levels of PRLR (35) and PRL stimulates
140 adult liver growth and regeneration (8, 34, 37), therefore we investigated whether
141 changes in liver growth during postnatal development associate with PRL serum
142 levels. In agreement with previous findings (38, 45), PRL serum concentration in

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143 the offspring was undetectable at PNW 1 and 2 but by PNW 4 PRL values reached
144 adult levels (7.34 ± 1.4 ng/ml) (Figure 2A). Therefore, undetectable and high
145 systemic values of PRL associate with the fall and rise in LBW occurring during
146 and after the first 2 PNW, respectively.

147 Because the long PRLR ($_L$PRLR) is the major isoform signaling all PRL
148 actions (4, 36), we evaluated the expression of the $_L$*Prlr* in the liver throughout
149 postnatal development. The mRNA levels of $_L$*Prlr* were barely detectable in
150 newborn animals, increased after birth, reached a peak at PNW 2, and declined to
151 stable levels thereafter (Figure 2B). Accordingly, the $_L$*Prlr* is upregulated and may
152 facilitate the action of PRL in liver at PNW 2.

153 Because circulating PRL is not detectable during the first 2 PNW, we
154 investigated whether the neonate liver produces PRL. We found that the liver
155 expresses the *Prl* mRNA and upregulates it by 20- and 10-fold during PNW 1 and
156 2, respectively (Figure 2C). In support to the production of PRL by the liver, the
157 PRL protein was measured by ELISA in the liver and at concentrations that were
158 higher at PNW 1 than at PNW 2 (Figure 2D). However, PRL was also measured at
159 PNW 4 when liver *Prl* mRNA values are minimal, although systemic PRL is high.

160 *Deletion of the PRLR modifies postnatal liver growth.* To study the effect of
161 PRL on postnatal liver development, we evaluated the liver and body weight and
162 the LBW in *Prlr-/-* mice relative to their wild type counterparts. There were no
163 differences at birth, implying that PRL does not affect prenatal liver and body
164 growth (Figure 3 A-C). However, in *Prlr-/-* mice the weight of the liver was reduced,
165 the body weight did not change, and the LBW was, thereby, smaller at PNW 1, 4,

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166 6, and 10 (Figure 3 A-C). In contrast, the liver and body weight of *Prlr-/-* mice
167 increased and the LBW was higher at PNW 2 (Figure 3A-C).

168 Reduced liver weight in the absence of the PRLR associated with lower liver
169 cell proliferation and angiogenesis, as indicated by corresponding variations in the
170 expression of genes encoding for the cell cycle progression protein, *Ccnd1* (Figure
171 3D) and for *Pecam1*, a specific marker of endothelial cells (Figure 3E). Both genes
172 were downregulated at PNW 1 and 4 but unmodified at PNW 2. We conclude that
173 PRL stimulates liver growth except at PNW 2 when it is inhibitory to both liver and
174 body weight.

175 *Deletion of the PRLR stimulates GH signaling at PNW 2.* In rodents, the first
176 2 weeks of postnatal life are defined as the GH-independent phase of body growth
177 and are characterized by GH signaling insensitivity in the liver (30). We reasoned
178 that PRL may inhibit liver and body growth during the first 2 PNW by promoting GH
179 liver insensitivity. To study this possibility, we evaluated the mRNA levels of GH
180 receptors (GHR) in the liver during postnatal development. The expression pattern
181 of the *Ghr* gene in wild-type mice was low at birth, higher at PNW 1, and maximal
182 at PNW 2 and 4 (Figure 4A). The pattern was similar in *Prlr-/-* mice except at PNW
183 1 when the *Ghr* mRNA levels were reduced. *Igf-1* is under the control of GH in the
184 liver and IGF-1 is a key mediator of GH-stimulated body growth (26). *Socs2* and *3*
185 are also induced by GH in the liver, where they inhibit GH-induced JAK2/STAT5b-
186 mediated *Igf-1* expression (1, 17, 18, 29). However, SOCS2 also enhances GH
187 action by binding and targeting SOCS3 for proteasome-dependent degradation
188 (41, 46). *Prlr-/-* mice exhibited lower and elevated *Igf-1* mRNA liver content at PNW
189 1 and 2, respectively, compared to wild-type controls (Figure 4B). The hepatic

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190 content of *Socs2* mRNA was higher at 2 weeks (Figure 4C), and that of *Socs3* was
191 lower at PNW 1 and 2 (Figure 4D) in *Prlr-/-* mice. There were no differences in *Igf-*
192 *1, Socs2, and Socs3* expression levels at birth and at PNW 4 between *Prlr-/-* and
193 *Prlr+/+* mice (Figure 4B-D).
194

195 **DISCUSSION**

196 PRL was reported to be a liver mitogen more than 30 years ago, when
197 pharmacological doses of the hormone increased liver DNA synthesis, mitosis, and
198 the LBW (8). Since then several reports have confirmed these findings (5, 7, 34,
199 36, 37, 48, 51), but the physiological meaning of the effect of PRL on liver growth is
200 unclear. Here, we show that PRL is required for normal liver growth throughout
201 postnatal development and unveil a novel mechanism by which PRL restricts liver
202 and body growth at PNW 2.

203 In rodents, the pituitary gland barely produces PRL after birth (22) and the
204 hormone is undetectable in the circulation throughout the first 2 PNW until weaning
205 (PNW 3), when a substantial increase in pituitary PRL secretion occurs (38, 45)
206 present findings). We found that the absence of systemic PRL during the first 2
207 PNW, can be compensated in the liver by the expression of the PRL mRNA and
208 protein. This is the first evidence that the liver produces PRL and, while the cell
209 type in the liver expressing the hormone needs to be investigated, this finding
210 suggests that local PRL functions as an autocrine/paracrine liver regulator when
211 circulating PRL is lacking. Alternatively, the milk contains high levels of PRL (12),
212 and it is possible that PRL from milk enters the pup and accumulates in the liver
213 during the first 2 PNW, when the gut is permeable to large molecules (10). In this

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214 regard, PRL was also measured in the liver at PNW 4, when hepatic *Prl* mRNA
215 levels are minimal. Because circulating PRL is high at PNW 4, the PRL measured
216 in the liver at this time could have originated from the incorporation of systemic
217 PRL into the liver. Different organs transfer and concentrate PRL from the
218 circulation, including the eye (3), the mammary gland (43), and the brain (6). From
219 these findings, we conclude that during early postnatal life the liver is under the
220 influence of PRL produced locally (by the liver) and of PRL in milk entering the
221 offspring, whereas systemic PRL (produced by the pituitary gland) influences the
222 liver thereafter.

223 Justifying the direct action of PRL in the liver, the hepatic $_{L}$*Prlr* mRNA is
224 hardly detectable after birth, and its levels peak at PNW 2 and decline to stable
225 levels thereafter. These findings are consistent with previous observations showing
226 that PRLR mRNA and binding sites increase in rat liver during postnatal
227 development (23). PRLRs exist in various molecular forms classified as long,
228 intermediate, and short due to the different length of their cytoplasmic domain (4).
229 The $_{L}$PRLR is considered the major isoform signaling all PRL actions, the
230 intermediate (absent in mice) transmits proliferation and survival signals, and the
231 short PRLR silences the effect of the long form (4). The upregulation of the $_{L}$*Prlr* in
232 neonatal liver implies that PRL can act directly on the liver during early postnatal
233 development.

234 By analyzing PRLR null mice we demonstrate that PRL is required for
235 normal postnatal liver and body growth. Consistent with previous observations in
236 adult animals, the absence of the PRLR confers a reduced liver mass (34). The
237 effect is subtle but similar to those of other regulators. For example, deletion of the

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238 yes-associated protein, a postnatal liver mitogen, results in a modest, albeit
239 significant, 8% reduction in LBW at PNW 4 (44), which is equivalent to the 7%
240 reduction that we observed in *Prlr-/-* mice. Likewise, small increases (≈10%) in
241 physiological liver growth follow the treatment with PRL (34) and other well-known
242 liver mitogens, such as hepatocyte growth factor (42). The reduction in liver mass
243 does not occur during prenatal development indicating that PRL and placental
244 lactogens are not involved in embryonic or fetal liver growth. Decreased liver
245 weight and LBW happen at PNW 1 and at PNW 4 to 10 and are supported by the
246 decreased expression of markers of cell proliferation and angiogenesis in the liver.
247 These findings agree with previous reports showing that PRL treatment in adult
248 rats stimulates the proliferation of hepatocytes and endothelial cells (34) and the
249 activity of transcription factors (AP-1, Jun, STAT3) involved in activating DNA
250 synthesis and cell proliferation (37). Furthermore, PRL acts on hepatocytes
251 isolated from adult rats or 3-week old rats to induce the expression of c-*myc* (51),
252 c-*fos*, c-*jun*, and c-*src* (5), or cell proliferation (48), respectively. The proliferative
253 effects of PRL on hepatocytes and endothelial cells involve the activation of JNK,
254 p38-MAPK, PKC, pp60$^{c\text{-}src}$, STAT3, STAT5, and heme oxygenase-1 pathways (5,
255 28, 37, 50). However, the question of whether changes in liver mass involve PRL-
256 induced effects on the size of hepatocytes needs to be addressed.

257 In contrast to previous findings, our study shows that PRL not only
258 stimulates but also restricts liver growth. The negative effect manifests at PNW 2,
259 when the liver and body weight and the LBW increase in *Prlr-/-* mice. These PRL
260 inhibitory actions have not been appreciated previously (15), perhaps because they

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261 only occur during the first 2 PNW, a period defined as the GH-independent growth
262 phase. GH is a major postnatal growth stimulator, except during fetal or early
263 postnatal development. GH-spontaneous mutants have normal weights at birth and
264 at PNW 2, after which time their growth retardation manifests (25). Likewise,
265 accelerated growth in transgenic GH mice commences at PNW 3 despite high
266 circulating GH levels at birth (31, 40). GH acts on the liver to stimulate somatic
267 growth (47) in part by inducing *Igf-1* transcription (27), the liver is the major source
268 of circulating IGF-1 (27) and, both, IGF-1 liver expression and circulating levels are
269 unresponsive to GH until after PNW 2 (31). Because of this timing, we explored
270 whether PRL inhibitory actions on liver and body growth could be due to
271 PRL-mediated stimulation of GH insensitivity. We found that PRLR deficiency did
272 not alter GH receptors in postnatal liver except at PNW 1, when *Ghr* mRNA levels
273 were reduced. This finding is consistent with the lower expression of the GH target
274 genes, *Igf-1* and *Socs3* in 1-PNW *Prlr-/-* mice, and imply that PRL promotes GH
275 signaling at this time. Nevertheless, the expression levels of *Igf-1* and *Socs2* were
276 enhanced in the liver of *Prlr-/-* mice at PNW 2, suggesting that PRL later promotes
277 GH insensitivity in the liver.

278 The opposite actions on GH signaling are consistent with the stimulatory
279 and inhibitory effects of PRL on LBW and LBW and body weight at PNW 1 and 2,
280 respectively, and may relate to the differential hepatic gene expression of *Socs2*
281 and *Socs3*. SOCS3 has clear inhibitory effect on GH signaling (1), but the role of
282 SOCS2 has been ambiguous. In vivo and in vitro observations have revealed both
283 inhibitory and stimulatory effects of SOCS2 on GH signaling. *Socs2* null mice (32)
284 and transgenic mice overexpressing *Socs2* (18) both exhibit an overgrowth

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285 phenotype. SOCS2 inhibits GH signaling when expressed at very low levels, and
286 higher concentrations of SOCS2 restore responsiveness to GH (14). It is
287 suggested that dual effects of SOCS2 depend on its concentration in the cell (14,
288 18). Therefore, we hypothesize that the upregulation and downregulation of *Socs2*
289 and *Socs3,* respectively, observed at PNW 2 in *Prlr-/-* mice, promote GH signaling
290 and lead to elevated *Igf-1* expression and increased liver and body weights. At
291 PNW1, decreased *Ghr* and *Socs3* transcription in *Prlr-/-* mice, could have resulted
292 in the observed attenuation of GH action (lower *Igf-1* expression and reduced liver
293 growth) due to lower levels of GH receptors unable to overcome the stimulatory
294 effect of reduced *Socs3*. These mechanisms need to be addressed by further
295 research.

296 *Perspectives and significance.*

297 Our work demonstrates that PRL is a physiological regulator of postnatal
298 liver development. While we show that the net effect of PRL is to stimulate liver
299 growth throughout postnatal development, we have also uncovered the ability of
300 PRL to restrict liver and body growth. Inhibition manifests at PNW 2, a period of
301 development characterized by the absence of circulating PRL, the hepatic
302 production of PRL and upregulation of the PRLR, and the liver insensitivity to GH.
303 We suggest that PRL contributes to the latter by attenuating hepatic GH-induced
304 *Igf-1* transcription via the upregulation and downregulation of *Socs2* and *Socs3*,
305 respectively. Similar mechanisms could operate to adjust liver mass throughout life
306 at times characterized by elevated PRL levels and may help improve liver health
307 under disease, warranting further investigation.

308

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313

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322

323 **DISCLOSURES**

324 No conflicts of interest, financial or otherwise, are declared by the authors.

325

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491 **FIGURE LEGENDS**

492 **Figure 1. Liver and body growth during postnatal development.** Liver (**A**) and
493 body (**B**) weights, and liver-to-body weight ratio (LBW) (**C**) in mice at birth
494 (postnatal week 0) and at postnatal weeks 1 to 10. Values are means ± SE. *n*
495 values are shown above or below graphs. P values are indicated only when
496 significant.

497

498 **Figure 2. The liver is under the influence of PRL throughout postnatal**
499 **development.** Serum PRL levels (A), liver expression of the long PRL receptor
500 ($_{L}$*Prlr*) mRNA (B), and liver expression of PRL mRNA and protein (C and D) in wild
501 type mice sacrificed at birth (postnatal week 0) and at postnatal weeks 1-10. PRL
502 levels in serum and liver were measured by ELISA, and mRNA values were
503 determined by quantitative RT-PCR using *Gapdh* and *CypA* as house-keeping
504 genes for normalization. Arbitrary units (AU). Values are means ± SE. *n* values are
505 shown above graphs. P values are indicated only when significant.

506

507 **Figure 3. Deletion of the PRL receptor alters postnatal liver growth.** Liver (**A**)
508 and body (**B**) weights, liver-to-body weight ratio (LBW) (**C**), and expression of
509 genes encoding for the proliferation (**D**) (cyclin D1, *Ccnd1*) and angiogenesis (**E**)
510 (platelet/endothelial cell adhesion molecule 1, *Pecam1*) markers in mice null (*Prlr-/-*
511) or not (*Prlr+/+*) for the PRL receptor at birth (postnatal week 0) and at postnatal
512 weeks 1 to 10. mRNA values were determined by quantitative RT-PCR using
513 *Gapdh* and *CypA* as house-keeping genes for normalization. Arbitrary units (AU).
514 Values are means ± SE. *n* values are shown inside or above bars. P values are

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515 indicated only when significant.

516 **Figure 4. Deletion of the PRL receptor modifies hepatic GH signaling.** Liver
517 expression of the GH receptor (*Ghr*) (**A**), *Igf-1* (**B**), *Socs2* (**C**), and *Socs3* (**D**) genes
518 in mice null (*Prlr-/-*) or not (*Prlr+/+*) for the PRL receptor at birth (postnatal week 0)
519 and at postnatal weeks 1, 2 and 4. mRNA values were determined by quantitative
520 RT-PCR using *Gapdh* and *CypA* as house-keeping genes for normalization.
521 Arbitrary units (AU). Values are means ± SE. *n* values are shown inside or above
522 bars. P values are indicated only when significant.

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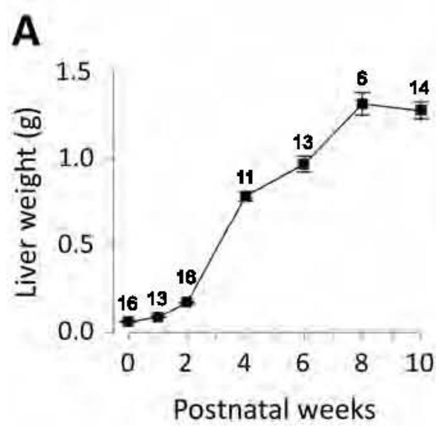

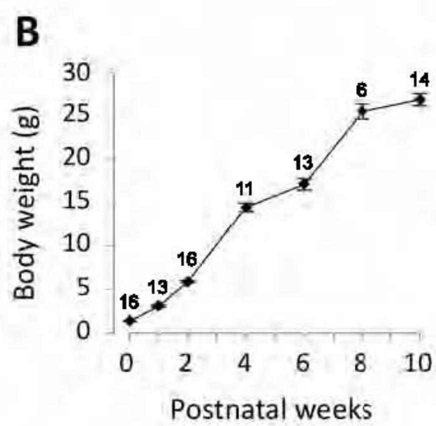

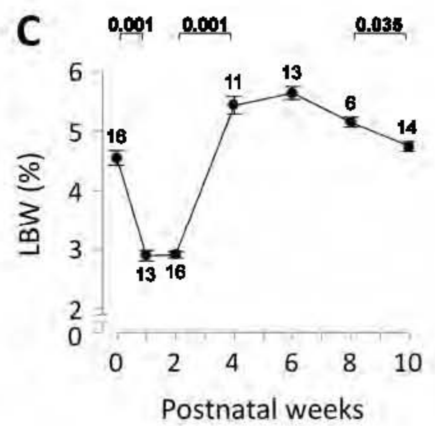

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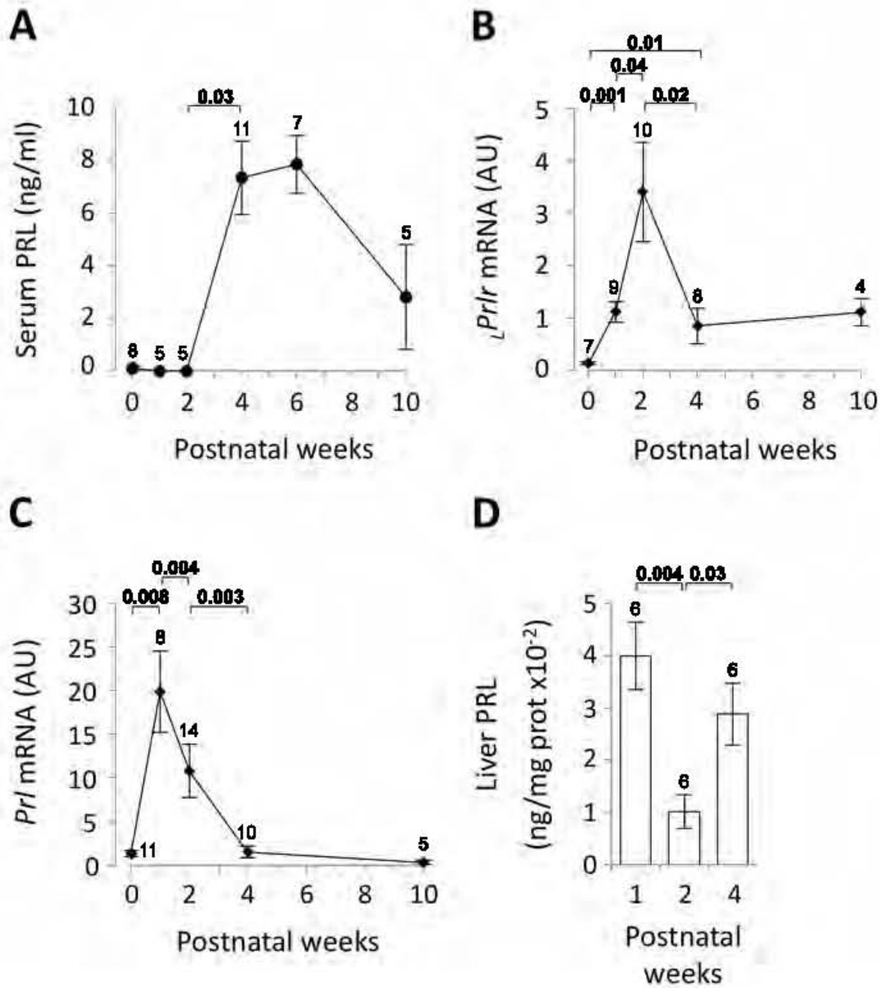

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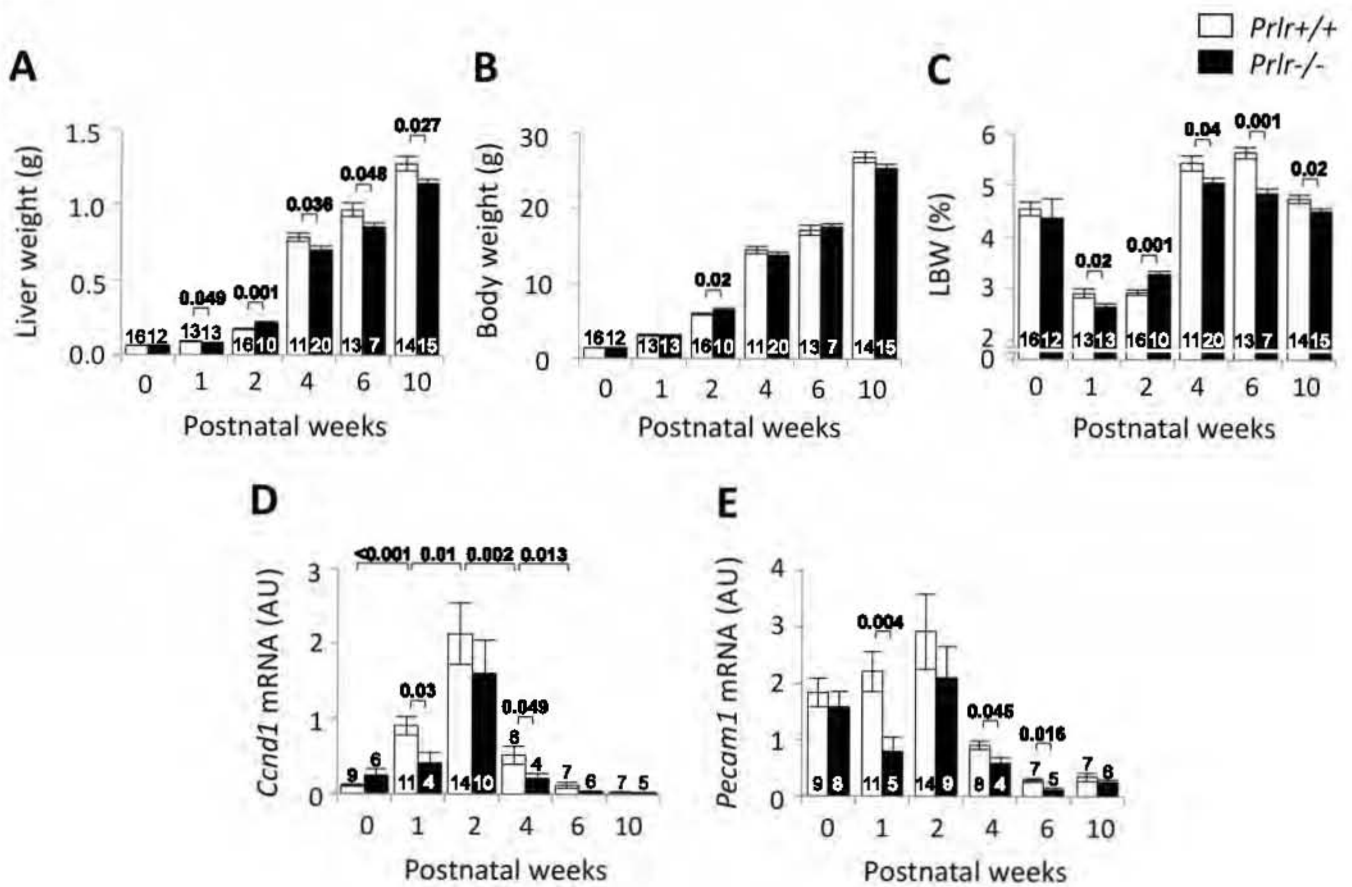

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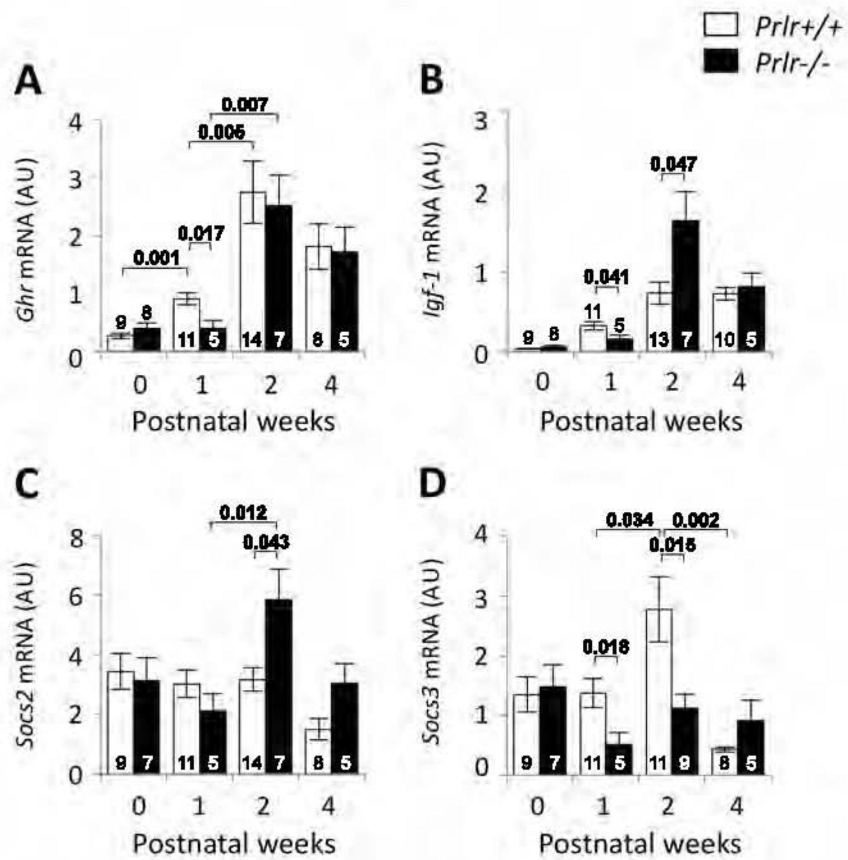

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