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Papel de la hormona de crecimiento en el desarrollo de enfermedad por hígado graso no alcohólico en un modelo celular *in vitro.*

TESIS

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DEDICATORIA

A mis grandes amores: Edmundo y Rogelio,
por todo su amor y comprensión.
Por ser mi motor para esforzarme cada día.
¡Los amo!

A mis padres, mis hermanas y mi tía,
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RESUMEN

La deficiencia de hormona de crecimiento (GH) se ha asociado a la presencia de esteatosis hepática, sin embargo los mecanismos moleculares encargados de mediar esta acción no han sido completamente explicados.

Objetivo: En este trabajo nos propusimos investigar el efecto de la GH sobre la acumulación lipídica en células HepG2 cultivadas *in vitro* en condiciones de esteatosis. Evaluamos la participación del factor de crecimiento similar a la insulina tipo 1 (IGF-1), así como de moléculas lipogénicas y lipolíticas.

Material y métodos: Cultivamos las células HepG2 en condiciones control y de esteatosis, inducidas mediante la administración de glucosa en una concentración 5.5 o 25 mmol/l respectivamente, durante 24 h. Posteriormente, las células de ambas condiciones fueron expuestas a distintas concentraciones de GH (0, 5, 10 ó 20 ng/ml) durante 24 h más. Cuantificamos el contenido de lípidos en las condiciones previamente descritas, así como los niveles de RNAm y de las proteínas: IGF-1, proteína de unión al elemento de respuesta a carbohidratos (ChREBP), proteína de unión al elemento de respuesta a esteroles 1c (SREBP1c), sintasa de ácidos grasos (FAS), carnitina palmitoil transferasa 1A (CPT1A), y receptor alfa activado por el proliferador de peroxisomas por el (PPAR-α) mediante RT-qPCR y western blot, respectivamente.
Los datos fueron analizados mediante ANOVA de una vía y el test post-hoc de Games-Howell.

Resultados: En el modelo de esteatosis, los hepatocitos HepG2 mostraron un incremento hasta dos veces mayor del contenido lipídico comparado con la condición control. Los niveles de RNAm y proteína de IGF-1 mostraron un incremento significativo en la condición control a 10 ng/ml de GH, mientras que en la condición hiperglucémica este efecto se suprimió.

La hiperglucemia también incrementó significativamente los niveles de RNAm y proteínas de ChREBP y FAS, no se demostró efecto sobre SREBP1c, CPT1A y PPAR-alfa. A la administración de GH se demostró la inhibición de ChREBP y FAS, aún en los hepatocitos HepG2 cultivados bajo condiciones de esteatosis.

Conclusiones: La hormona de crecimiento disminuye la esteatosis inducida por altas dosis de glucosa en células HepG2 mediante la supresión de la lipogénesis de novo disminuyendo la expresión de ChREBP y FAS.

ABSTRACT

Objective: Growth hormone (GH) deficiency has been associated with increased steatosis but the molecular mechanism has not been fully elucidated. We investigated the effect of GH on lipid accumulation of HepG2 cells cultured on an in vitro steatosis model and examined the potential involvement of insulin-like growth factor 1 (IGF-1) as well as lipogenic and lipolytic molecules.

Methods: Control and steatosis conditions were induced by culturing HepG2 cells with 5.5 or 25 mmol/l glucose for 24 h, respectively. Afterward, cells were exposed to 0, 5, 10 or 20 ng/ml GH for another 24 h. Lipid content was quantified as well as mRNA and protein levels of IGF-1, carbohydrate responsive element-binding protein (ChREBP), sterol regulatory element-binding protein 1c (SREBP1c), fatty acid synthase (FAS), carnitine palmitoyltransferase 1A (CPT1A), and peroxisome proliferator-activated receptor alpha (PPAR-alpha) by RT-qPCR and western blot, respectively. Data were analyzed by one-way ANOVA and the Games-Howell post-hoc test.

Results: In the steatosis model, HepG2 hepatocytes showed a significant 2-fold increase in lipid amount as compared to control cells. IGF-1 mRNA and protein levels were significantly increased in control cells exposed to 10 ng/ml GH, whereas high glucose abolished this effect. High glucose also significantly increased both mRNA and protein of ChREBP and FAS without having effect on SREBP1c, CPT1A and PPAR-alpha. However, GH inhibited ChREBP and FAS production, even in HepG2 hepatocytes cultured under steatosis conditions.

Conclusions: Growth hormone ameliorates high glucose-induced steatosis in HepG2 cells by suppressing de novo lipogenesis via ChREBP and FAS down-regulation.

ABREVIATURAS

NAFLD	Enfermedad de hígado graso no alcohólico (*Non-Alcoholic Fatty Liver Disease*)
NASH	Esteatohepatitis no alcohólica (*Non-Alcoholic SteatoHepatitis*)
GH	Hormona de crecimiento (*Growth Hormone*)
VLDL	Lipoproteínas de muy baja densidad (*Very Low Density Lipoprotein*)
DNL	Lipogénesis *de novo* (*De Novo Lipogenesis*)
FATP	Proteína transportadora de ácidos grasos (*Fatty Acid Transport Protein*)
CD36	Grupo de diferenciación 36 (*Cluster of Differentiantion 36*)
PPAR-γ	Receptor gama activado por el proliferador de peroxisomas (*Peroxisome Proliferator-Activated Receptor gamma*)
ACC	Acetil CoA carboxilasa (*Acetyl CoA-Carboxylase*)
FAS	Sintasa de ácidos grasos (*Fatty Acid Synthase*)
ChREBP	Proteína de unión al elemento de respuesta a carbohidratos (*Carbohydrate Response Element Binding Protein*)
SREBP1-c	Proteína de unión al elemento de respuesta a esteroles (*Sterol Regulatory Element-Binding Protein 1-c*)
CPT1A	Carnitina palmitoil transferasa (*Carnitine Palmitoyl Transferase 1A*)
PPAR-α	Receptor alfa activado por el proliferador de peroxisomas (*Peroxisome Proliferator-Activated Receptor alpha*)
ATP	Adenosín trifosfato
MTTP	Proteína microsomal de transferencia de triglicéridos (*Microsomal Triglyceride Transfer Protein*)
GLP-1	Péptido similar a glucagon 1 (*Glucagon Like Peptide 1*)
DMEM	Medio de cultivo de Eagle modificado por Dulbecco (*Dulbecco's Modified Eagle Medium)*
PCR	Reacción en cadena de la polimerasa (*Polymerase Chain Reaction*)
RT-qPCR	Reacción en cadena de la polimerasa en tiempo real con transcriptasa inversa (Quatitave Reverse Transcription -*Polymerase Chain Reaction*)

IGFBP3 Proteína 3 de unión al factor de crecimiento parecido a la insulina (*Insulin Growth Factor Binding Protein 3*)

PBS Solución amortiguadora de sales de fosfato fostato salino (*Phosphate Buffered Saline*)

INTRODUCCIÓN

1. Enfermedad de hígado graso no alcohólico (NAFLD). Definición.

La enfermedad de hígado graso no alcohólico (NAFLD - non alcoholic fatty liver disease, por sus siglas en inglés) es un padecimiento que comprende un espectro amplio de cambios histopatológicos en el hígado, que van desde la acumulación de grasa intrahepática hasta un proceso necroinflamatorio que puede progresar a cirrosis hepática y hepatocarcinoma celular (1,2).

De forma adicional, NAFLD es un síndrome clínico patológico que ocurre en ausencia de otras causas secundarias de infiltración grasa como infección viral crónica, uso de fármacos inductores de esteatosis (como amiodarona y tamoxifeno), enfermedades autoinmunes, enfermedades por depósito (hemocromatosis y enfermedad de Wilson), o antecedente de abuso en el consumo de alcohol (> 20g/día en mujeres o > 30g/día en hombres) (3,4).

La definición de NAFLD se da como un diagnóstico de exclusión y el estándar de oro es la biopsia hepática, que permite documentar histopatológicamente la presencia de esteatosis en más del 5% de hepatocitos, así como otros cambios histopatológicos como balonización de hepatocitos, presencia de infiltrado inflamatorio y necrosis (5).

Si bien la historia natural de la patología narra una progresión de NAFLD hacia NASH (NASH - non alcoholic steatohepatitis, por sus siglas en inglés), este no necesariamente es un fenómeno lineal, se estima que un individuo puede cursar con esteatosis durante 14 años, en promedio, para finalmente desarrollar fibrosis (2).

La progresión de esta patología incrementa el riesgo de desarrollar cirrosis hepática e insuficiencia hepática que ameritará trasplante hepático. Fases avanzadas de la patología aumentan significativamente el desarrollo de hepatocarcinoma celular y mortalidad asociada a estas entidades (6).

1.1 Epidemiología

Actualmente, la NAFLD es una de las principales hepatopatías crónicas a nivel mundial y se ha convertido en un reto para la salud pública, ya que representa también una de las principales causas actuales de trasplante hepático en Estados Unidos (7). La prevalencia descrita en población general es de 25%, con una mayor prevalencia en Medio Oriente y Sudamérica (31.8% y 30.4% respectivamente) (2). En México la prevalencia de NAFLD es de hasta 17.05% en individuos adultos asintomáticos, y en la población pediátrica con sobrepeso / obesidad representa el 12.6% (8,9).

Durante las últimas tres décadas, la prevalencia de NAFLD a nivel mundial se ha incrementado de manera progresiva, a la par de la epidemia de diabetes y obesidad. En pacientes con grados avanzados de obesidad, la presencia de NAFLD puede ser mayor al 90%, y en el caso de pacientes con *Diabetes Mellitus* tipo 2 llega a ser mayor al 76% (4).

Los pacientes con NAFLD incrementan sus factores de riesgo de mortalidad al asociarse a la presencia de otras enfermedades crónicas concomitantes, principalmente de origen cardiovascular, carcinoma hepatocelular y otras patologías hepáticas relacionadas (10,11).

1.2 Factores de riesgo

Existen múltiples factores de riesgo que se han descrito para el desarrollo de NAFLD, sin embargo, entre los más frecuentes se encuentran el género masculino, el ser de origen latino o asiático, la predisposición genética y la presencia de síndrome metabólico (2).

El síndrome metabólico, ya sea en conjunto o a través de sus componentes en lo individual (circunferencia de cintura, dislipidemia, hiperglucemia e hipertensión arterial), son las variables más frecuentemente asociadas a NAFLD. Esta asociación ha sido tan constante que se considera a NAFLD como la manifestación hepática del síndrome metabólico (11,12). La población con *Diabetes Mellitus* tiene una prevalencia de NAFLD mayor al 70% y una mayor progresión a NASH (11,13).

El hecho de que exista gran variabilidad étnica ha llevado a proponer una predisposición genética para esta patología. Una de las propuestas más sólidas ha sido la identificación de

la variante polimórfica I148-M del PNPLA3, que interfiere con la lipólisis de cuerpos lipídicos a nivel hepático y que ha sido más frecuentemente encontrada en población mexicana (14). Sin embargo, esto no es 100% determinante, pues es necesario tomar en cuenta la predisposición genética en el contexto de factores medio-ambienteales como el acceso a una alimentación hiperenergética acompañada de poca actividad física y factores socioeconómicos particulares (2,15).

Con menor frecuencia, pero sin menor importancia, también se ha documentado la asociación entre NAFLD y algunas endocrinopatías, como el hipogonadismo en varones, hiperandrogenismo en mujeres con síndrome de ovario poliquístico e hipercortisolismo endógeno o exógeno (10). La presencia de hipotiroidismo de igual forma se asocia con NAFLD, y es relevante hacer notar que estos hallazgos han llevado a pensar que los mecanismos hormonales pudieran tener una participación fisiopatológica, a tal grado que actualmente se ha planteado como una posible estrategia terapéutica un agonista hepático específico del receptor tiroideo (16).

De igual forma, se ha observado un dimorfismo sexual en la prevalencia de NAFLD y de sus complicaciones hepáticas, siendo más frecuente su presentación en el género masculino, y en mujeres posmenopáusicas, esta observación ha llevado a hipotetizar la posibilidad de que los estrógenos podrían tener un papel esencial como factor protector en esta hepatopatía (17–19).

La deficiencia de hormona de crecimiento (GH - growth hormone, por sus siglas en inglés) se ha asociado también con una mayor prevalencia de NAFLD. La idea de que la GH pudiera tener un papel importante en la fisiopatología de la NAFLD ha dado lugar a la ejecución de estudios clínicos y experimentales en modelos animales que han sido tratados con GH como terapia de sustitución hormonal, demostrando mejoría de las condiciones metabólicas y de NAFLD (20–23).

1.3 Fisiopatología

La patogénesis de la NAFLD es multifactorial. A finales del siglo pasado la patogénesis de la NAFLD y su progresión encontró una posible explicación en la hipótesis del "doble

golpe", propuesta por Day y James en 1998. La hipótesis del "doble golpe" proponía que una primera fase o "primer golpe" estaba dada por la infiltración hepática de triglicéridos mediada por resistencia a la insulina, lo cual hacía al tejido hepático susceptible a otros factores de daño como estrés oxidativo, depleción de ATP y endotoxinas. De forma colectiva, estos factores podían promover el "segundo golpe" que favorecería la progresión de esteatosis macrovesicular a NASH (3,24). La progresión de esta hepatopatía involucra un daño hepático gradual asociado a la apoptosis de hepatocitos, inflamación y fibrogénesis, que a su vez predispone a cirrosis, falla hepática y carcinogénesis (25).

A medida que se ha adquirido mayor conocimiento sobre la fisiopatología de NAFLD, el concepto de la hipótesis del “doble golpe” ha tenido que evolucionar, y actualmente la progresión de NAFLD se explica más bien bajo la visión de la teoría de golpes múltiples y en paralelo (26,27). Se atribuye como insulto inicial a la resistencia a insulina, que lleva a una mayor síntesis y captación de ácidos grasos libres que se almacenarán en el tejido hepático en forma de triglicéridos, dando lugar a la esteatosis. Y en el segundo insulto, múltiples factores de daño actúan de manera superpuesta en la progresión de NAFLD hacia estadios más avanzados de la enfermedad, incluyendo estrés oxidativo, inflamación local y exógena, apoptosis, estímulos profibróticos, entre otros. Actualmente se reconoce que el desarrollo y la presencia de NASH puede ocurrir incluso sin el antecedente de esteatosis (25).

Este cambio de paradigma en cuanto a la progresión en NAFLD puso en evidencia que los principales factores involucrados en el daño inflamatorio del tejido hepático son los ácidos grasos libres y no la acumulación de triglicéridos al interior de los cuerpos lipídicos. El metabolismo hepático de los ácidos grasos libres lleva a la formación de metabolitos tóxicos como el diacilglicerol y las ceramidas, responsables de la generación de estrés oxidativo, inflamación y lesión al parénquima hepático (28).

La acumulación de ácidos grasos libres en NAFLD resulta de un desequilibrio entre el aumento en la disponibilidad de ácidos grasos libres debido al incremento en la síntesis y captación de los mismos por el tejido hepático, así como una reducción de la beta-

oxidación mitocondrial, la exportación de VLDL (lipopoteínas de muy baja densidad - very low density lipoprotein, por sus siglas en inglés) y la lipofagia (25).

1.3.1 Mecanismos de acumulación lipídica en NAFLD

Existen 3 fuentes de ácidos grasos que contribuyen a la infiltración de triglicéridos en NAFLD: el 59% viene de los ácidos grasos circulantes, 26% proviene de la generación de ácidos grasos a partir de precursores no lipídidos (glucosa y fructosa) por medio de la lipogénesis *de novo* (DNL, por sus siglas en inglés); y 14% de la dieta (10).

El tejido hepático es el regulador central de la homeostasis lipídica, ya sea sintetizando nuevos ácidos grasos, participando en su exportación y en la distribución hacia otros tejidos para dar respuesta a la demanda energética (29).

Todo el proceso de balance de lípidos en el tejido hepático es un proceso complejo en el que interactúan factores hormonales, receptores nucleares y factores de transcripción; la alteración de una o más de estas vías puede precipitar una mayor acumulación de lípidos en el tejido hepático desencadenando NAFLD (30).

La regulación del metabolismo de lípidos a nivel hepático está dada por 4 principales mecanismos: la captación de lípidos circulantes, la DNL, la oxidación de ácidos grasos (b-oxidación) y la exportación VLDL (29–31) (Figura 1).

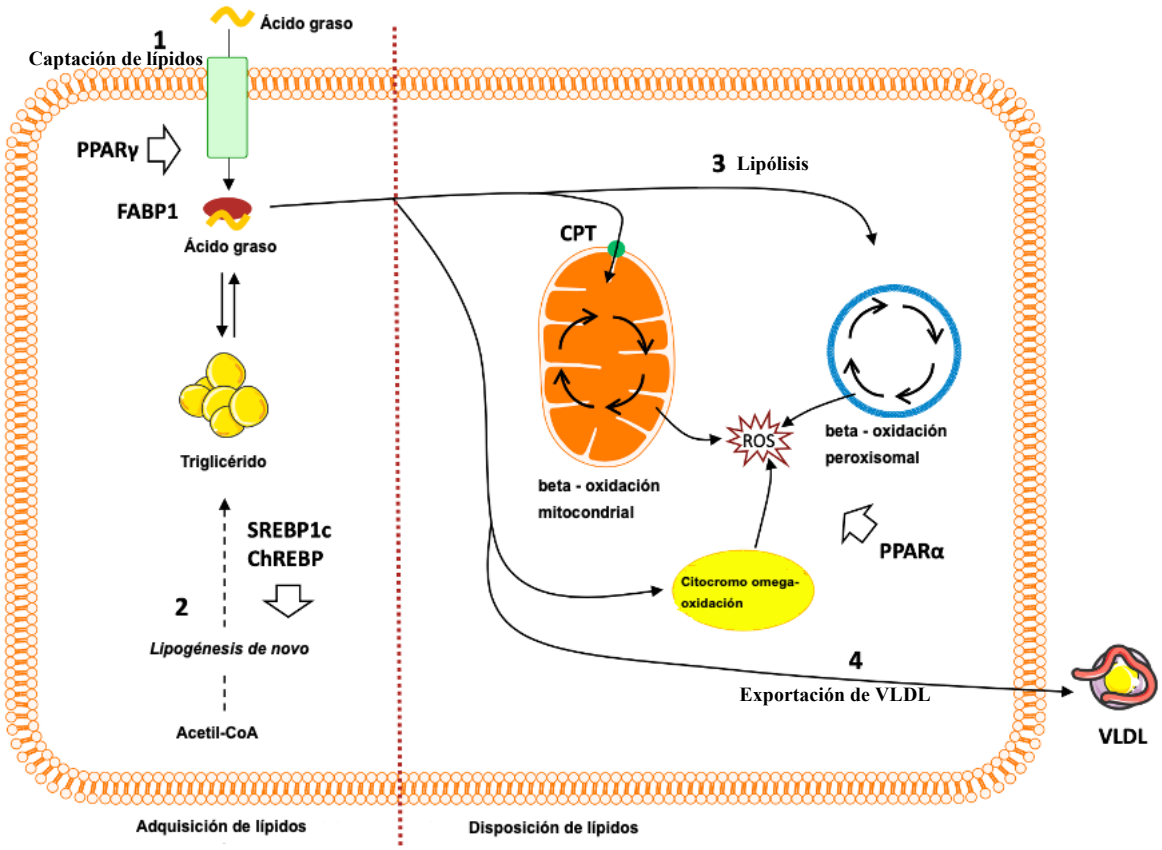

Figura 1. Vista general del metabolismo de lípidos en el hepatocito. (1) Captación de lípidos circulantes facilitado por transportadores de ácidos grasos específicos localizados en la membrana plasmática del hepatocito. (2) La lipogénesis *de novo* covierte acetil-CoA a nuevos ácidos grasos que subsecuentemente se esterifican formando triglicéridos; este proceso es regulado por dos factores de transcripción principales: SREBP1c (Sterol Regulatory Element-Binding Protein 1-c) y ChREBP (Carbohydrate Response Element Binding Protein). (3) La oxidación de ácidos grasos, que es controlada por PPARα (Peroxisome Proliferator-Activated Receptor alpha) y reduce los lípidos hepáticos utilizándolos como fuente energética. (4) El hígado puede exportar lípidos a través de partículas de VLDL (Very Low Density Lipoprotein). (Imagen y pie de figura modificado de Højland Ipsen y cols. 2018).

1.3.1.1 Captación de ácidos grasos por el tejido hepático

La captación de los ácidos grasos por el hígado depende en mayor parte de los transportadores de ácidos grasos, los más predominantes son las proteínas de transporte mediadas por ácidos grasos (FATP - fatty acid transport protein, por sus siglas en inglés), el grupo de diferenciación 36 (CD36, por sus siglas en inglés) y las caveolinas localizadas en la membrana plasmática del hepatocito (30,32). Se han descrito hasta 6 isoformas de FATP (desde FATP2 hasta FATP5, entre otras). En estudios realizados en modelos animales se ha observado que en el ratón knockout de los genes codificantes de FATP2 y FATP5 la

captación de ácidos grasos a nivel hepático disminuye, reduciendo el contenido de triglicéridos y por tanto revirtiendo la esteatosis. Estos hallazgos indican que la captación de lípidos mediada por FATP juega un papel relevante al facilitar la esteatosis hepática (33,34). En concordancia con lo reportado en modelos animales, en estudios clínicos realizados en pacientes adolescentes con NASH se ha observado una mayor expresión de los genes FATP2 y FATP5 al contrastarse con adolescentes sanos (35).

CD36 es una proteína con actividad de translocasa que facilita el transporte de ácidos grasos de cadena larga y a su vez es regulado por PPAR-γ, el receptor pregnano X y el receptor hepático X. Tanto en estudios realizados en modelos animales como en seres humanos, se ha documentado un incremento en la expresión de CD36 en pacientes con NAFLD y NASH (30,35).

1.3.1.2 Lipogénesis

El hígado es uno de los órganos más versátiles orquestando el anabolismo y catabolismo de macromoléculas. La adaptación metabólica en estado de ayuno o posprandial está regulada tanto por los nutrimentos ingeridos como por señales hormonales. Durante el estado posprandial la glucosa se almacena en forma de glucógeno en el tejido hepático, sin embargo, cuando la ingesta de glucosa es excesiva, el almacenamiento de energía se da también en forma de ácidos grasos a través de la vía metabólica de la DNL (31,36).

En condiciones normales un almacenamiento excesivo tanto de glucosa como de lípidos es parte de la respuesta metabólica adaptativa, sin embargo, un exceso en la activación de la DNL se ha asociado con la presencia de NAFLD. El proceso de la DNL se divide en tres procesos secuenciales: la síntesis de ácidos grasos, la elongación de los ácidos grasos, y su integración en triglicéridos.

El metabolismo de la glucosa por glucólisis conlleva la producción de metabolitos intermedios como el gliceraldehído 3 fosfato y la dihidroacetona fosfato; estos metabolitos pueden interconvertirse o trasformarse a su vez en piruvato. El piruvato tiene la capacidad de ingresar a la mitocondria y transformarse en acetil coA para participar en el ciclo de los ácidos tricarboxílicos (ciclo de Krebs) para la producción de energía. Cuando el

almacenamiento energético está completo, los productos intermediarios se acumulan y el citrato se transporta de regreso al citoplasma donde es convertido a acetil-CoA. El citrato es una activador alostérico de la acetil CoA carboxilasa (ACC, por sus siglas en inglés) que a su vez convierte acetil CoA a malonil CoA (principal fuente de carbonos para la síntesis endógena de ácidos grasos). La sintasa de ácidos grasos (FAS, por sus siglas en inglés) hace uso de la malonil CoA para elongar la cadena de acilos de los ácidos grasos, formando palmitato como uno de los principales productos de la síntesis de ácidos grasos (37). (Figura 2).

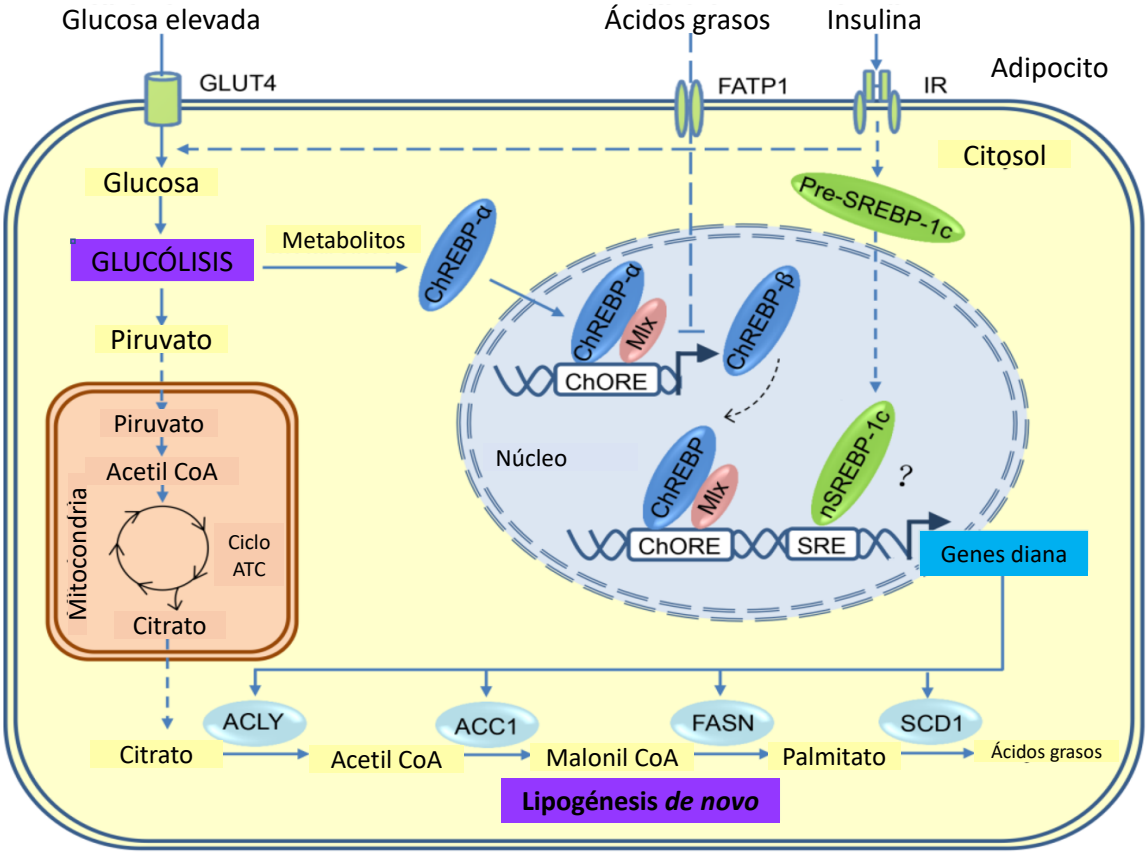

Figura 2. Activación transcripcional de la DNL en adipocitos en respuesta a una dieta alta en azúcares y grasas. Después del consumo de carbohidratos, una parte de la glucosa es procesada mediante glucólisis en el citosol, como uno de los productos finales se obtiene piruvato que se internaliza en la mitocondria como sustrato del ciclo de los ácidos tricarboxílicos (ciclo de Krebs). El citrato es un producto intermedio del ciclo de Krebs, al exportarse al citosol sirve de sustrato para la DNL. La regulación de la lipogénesis a nivel transcripcional está dada por ChREBP, el ChREBP-a se une al elemento de respuesta a carbohidratos (ChoRE) y promueve otros genes lipogénicos como ACLY (ATP-citrato liasa), ACC1, FASN (sintasa de ácidos grasos) y la SCD1 (stearoil-CoA desaturasa-1) y ChREBP-b. SREBP-1 (sterol regulatory element-binding protein-1) es otro factor de transcripción para lipogénesis mediada por insulina. (Figura y pie de figura modificado de Song y cols, 2018) (38)

Cuando la actividad de la DNL es muy elevada, la malonil CoA se acumula e inhibe la carnitina palmitoiltransferasa (CPT1A), enzima limitante del transporte y utilización de ácidos grasos en la mitocondria (37).

Una vez que se han sintetizado y elongado las cadenas de ácidos grasos, éstos pueden ser esterificados en una estructura de glicerofosfato para integrarse en triglicéridos. Durante la última fase de la síntesis de triglicéridos la diacilglicerol aciltransferasa convierte los diacilgliceroles en triglicéridos, este proceso es crítico, ya que la acumulación de diacilgliceroles en el tejido hepático está asociado con resistencia a insulina. Este mecanismo tiene un sustento clínico pues en pacientes con NAFLD se observa un incremento en la concentración intrahepática de diacilgliceroles (39).

El uso de trazadores con isótopos estables en estudios dinámicos ha permitido observar que los pacientes con NAFLD tienen un incremento muy importante en la actividad de la DNL aún en estado de ayuno (40). La importancia de la DNL en la fisiopatología de la NAFLD se sustenta además en estudios clínicos en los que se muestra que en promedio, hasta un 26% de los triglicéridos intrahepáticos en pacientes con NAFLD provienen de la DNL aunado a una aparente incapacidad para regular la DNL de un estado de ayuno a un estado posprandial, esta información parece indicar que la DNL tiene un papel central en la acumulación lipídica en pacientes con NAFLD (30,41).

La DNL se regula a través de dos principales factores de transcripción: la proteína de unión al elemento de respuesta a esterol (SREBP1c, por sus siglas en inglés) y la proteínas de unión al elemento de respuesta a carbohidratos (ChREBP, por sus siglas en inglés). SREBP1c es activado por insulina y por el receptor hepático X alfa; mientras que el ChREBP es activado directamente por carbohidratos. En pacientes con NAFLD y resistencia a insulina se ha documentado una mayor expresión de SREBP1c, lo que hace pensar que la resistencia a insulina a nivel hepático es selectiva, contribuyendo a incrementar la actividad de la DNL y por tanto a la infiltración lipídica del tejido hepático (42). A su vez SREBP1c contribuye a una mayor resistencia a insulina, ya que al incrementar la actividad lipogénica se empiezan a acumular productos intermedios que

pueden ser dañinos para el metabolismo, como los diacilgliceroles que interfieren con la vía de señalización de la insulina (28,43).

En el caso de ChREBP1c que como se mencionó antes es activado directamente por carbohidratos (glucosa), en estudios experimentales realizados en modelos animales en los que se ha generado *knockout* del gen de ChREBP1c existe una disminución de hasta un 65% de la DNL a nivel hepático comparado con ratones control, además de intolerancia a hidratos de carbono simples. Si bien el knockout de ChREBP1c en modelos animales disminuye la infiltración lipídica hepática, se ha observado de forma simultánea un incremento en la síntesis de colesterol y una mayor citotoxicidad, por lo que pareciera que ChREBP1c tiene más bien un efecto citoprotector, favoreciendo la acumulación de grasa neutra y limitando los niveles de colesterol libre que potencialmente incrementan el daño hepático (44).

En algunos estudios clínicos se ha demostrado que en pacientes con NAFLD el ChREBP está regulado a la baja comparado con sujetos sanos, mientras que SREBP1c principalmente en pacientes con estadios más avanzados de NAFLD y NASH, se encuentra sobreexpresado, siendo este gen el responsable de activar otros genes de la DNL como ACC1 y FAS, que además de contribuir a la infiltración de grasa intrahepática confieren una mayor vulnerabilidad para hepatotoxicidad (45).

1.3.1.3 Lipólisis

El tejido adiposo, en el estado posprandial, responde al estímulo de la insulina almacenando lípidos en su citoplasma, e inhibiendo a su vez la lipólisis de triglicéridos al interior del adipocito. En la condición de resistencia a insulina, el tejido adiposo disfunciona y pierde la capacidad de inhibir la lipólisis, por lo que incrementa la liberación de ácidos grasos libres hacia la circulación, de donde son captados y almacenados por el hígado (46).

La activación de PPAR-α promueve la transcripción de una serie de genes relacionados con la oxidación de ácidos grasos a nivel mitocondrial, así como en peroxisomas y citocromos, promoviendo de esta forma la reducción de ácidos grasos a nivel hepático (47–49). En

estudios clínicos no se ha identificado una diferencia en la expresión PPAR-α entre los pacientes con esteatosis y controles sanos, pero sí en los pacientes con NASH, en los que se identificó una menor expresión de PPAR-α que a su vez disminuye aún más con un mayor grado de actividad y estadio de fibrosis (50).

La oxidación de ácidos grasos se lleva a cabo principalmente en la mitocondria y está regulada primordialmente por el receptor alfa activado por el proliferador de peroxisomas (PPAR-α, por sus siglas en inglés). Este mecanismo le permite a la célula obtener sustratos energéticos como el adenosín trisfosfato (ATP) cuando las concentraciones de glucosa son bajas (30,49,51). El ingreso de los ácidos grasos a la mitocondria para el proceso de β-oxidación depende de la carnitina palmitoiltransferasa 1 (CPT1, por sus siglas en inglés) situada en la membrana mitocondrial externa (49). La β-oxidación en la mitocondria tiene una limitada capacidad para la oxidación de ácidos grasos de cadena muy larga, por lo que se apoya de la β-oxidación que se da en los peroxisomas. Al alcanzarse una importante sobrecarga lipídica, se activa la ω-oxidación en los citocromos (47,48). Si bien se logra el objetivo que es la oxidación de los ácidos grasos, estas vías metabólicas no habituales son generadoras de una gran cantidad de especies reactivas de oxígeno, estrés oxidativo y ácidos dicarboxílicos tóxicos que potencialmente promueven la inflamación y la progresión de la NAFLD a estadios avanzados (47).

La oxidación lipídica alterna contribuye al deterioro del DNA y la disfunción mitocondrial generando un círculo vicioso al promover la utilización de vías alternas de oxidación de ácidos grasos, trayendo consigo un mayor estrés oxidativo y daño hepático (52).

1.3.1.4 Exportación de lípidos

Además de la oxidación de ácidos grasos, la exportación de triglicéridos es la otra forma de disminuir el contenido de lípidos en el tejido hepático. Dada su naturaleza hidrofóbica, la única forma de exportar triglicéridos es en forma de partículas VLDL empaquetados junto a otras partículas de colesterol, apoproteínas y fosfolípidos (30). La apolipoproteína B100 (apoB100) y la proteína microsomal de transferencia de triglicéridos (MTTP, por sus siglas en inglés) son los principales componentes de la secreción de VLDL hepática y del

mantenimiento de la homeostasis lipídica en el hígado (46). La transcripción de MTTP también es regulada por PPAR-α, y a su vez, el incremento de MTTP se acompaña de una mayor secreción de apoB100, por lo que PPAR-α no sólo ejerce su efecto catabólico mediante la oxidación de ácidos grasos, sino también lo hace a través de la regulación del metabolismo de lipoproteínas. La secreción de VLDL y de triglicéridos VLDL (VLDL-TG, por sus siglas en inglés) está incrementada en pacientes con NAFLD, y aunque pareciera que este mecanismo podría contribuir a la regulación de la esteatosis, la exportación de VLDL tiende a estabilizarse cuando el contenido de grasa hepática es mayor al 10%, por lo cual, llega un momento en que la esteatosis sobrepasa la capacidad compensatoria de este mecanismo, haciéndolo insuficiente para lograr el equilibrio en el contenido de grasa intrahepática (53).

1.4 Tratamiento

No existe actualmente terapia farmacológica alguna aprobada específicamente para el tratamiento de NAFLD. Uno de los principales factores limitantes del desarrollo de nuevos tratamientos, es la falta de modelos predictivos preclínicos que puedan simular la enfermedad humana desde los cambios histológicos, la fisiopatología y sus alteraciones metabólicas. Estas barreras limitan la comprensión y la definición de un blanco terapéutico específico ante la complejidad de la NAFLD (54).

Partiendo del hecho de que la mayoría de los pacientes con lesión histopatológica variable de NAFLD tienen otras patologías crónico-degenerativas como obesidad, *Diabetes Mellitus* tipo 2 e hipertensión, actualmente se propone un manejo integral y holístico personalizado para el contexto de cada paciente, que parte en todos los casos de una modificación de estilos de vida incluyendo la incorporación de hábitos dietéticos sanos y mayor actividad física. Este abordaje busca además disminuir las comorbilidades que incrementan el riesgo de mortalidad en estos pacientes, principalmente factores de riesgo cardiovascular y metabólicos. Todas estas estrategias buscan de manera indirecta mejorar a su vez la condición hepática y no necesariamente se establece de forma exclusiva una terapia hepatocéntrica (10)

Como se mencionó anteriormente, una de las primeras líneas de tratamiento en NAFLD se basa en la pérdida de peso a través de la modificación de los estilos de vida. Estas medidas han demostrado mejoría en la esteatosis, resistencia a insulina e inflamación asociadas con NAFLD (55). En ensayos clínicos aleatorizados, una pérdida ponderal de al menos 7% disminuye la muerte celular y la inflamación en NASH, y en sujetos con pérdida ponderal mayor al 10%, se ha demostrado mejoría histológica en el grado de fibrosis (56). En cuanto a la actividad física no existe un consenso con respecto a la cantidad o tipo de ejercicio que brinda un mayor beneficio para pacientes con NAFLD y en general se extrapolan las recomendaciones de la American Heart Association para pacientes de riesgo cardiovascular (57).

Como se mencionó antes, la primera línea de tratamiento consiste en la modificación de los estilos de vida. Es importante reconocer que algunos pacientes tendrán algún grado de fibrosis y la meta en pérdida ponderal mayor al 10% puede ser un objetivo difícil de alcanzar y mantener, por lo que basados en el conocimiento actual de la fisiopatología, se han hecho algunas propuestas de tratamiento basados en posibles blancos terapéuticos y de esa forma modificar el curso de la enfermedad. Entre los tratamientos propuestos se encuentran los sensibilizadores de insulina como las tiazolidinedionas (58,59); tratamiento con análogos del péptido similar a glucagon 1 (GLP-1, por sus siglas en inglés) (60); antioxidantes como la vitamina E (58,61); moduladores de la inflamación y la fibrosis como la pentoxifilina, agentes antifibróticos y moduladores del metabolismo de lípidos (57).

La gran diversidad de propuestas farmacológicas basadas en posibles dianas terapéuticas que hasta el momento no permiten formalizar un estándar de tratamiento, pone en evidencia la necesidad de continuar el estudio de los mecanismos fisiopatológicos de la NAFLD.

2. Hormona de crecimiento (GH)

La GH es una hormona peptídica de 191 aminoácidos que se sintetiza en la adenohipófisis por las células somatotropas. A la GH se le atribuye principalmente la promoción del

crecimiento lineal durante la infancia; sin embargo la GH también tiene un papel fundamental en la regulación del metabolismo y en la composición corporal, entre otras acciones y, por lo tanto, sigue teniendo una gran relevancia en la vida adulta (62).
La hormona de crecimiento es una hormona pleiotrópica y tiene receptores en distintos tejidos, sin embargo, su principal acción es ejercida a nivel hepático, estimulando la producción del factor de crecimiento similar a la insulina tipo 1 (IGF-1, por sus siglas en inglés), que se reconoce actualmente como el principal efector de las acciones metabólicas y mitogénicas de la GH, es esta interacción la que se denomina eje GH/IGF-1 (63). La alta concentración tanto de GH como de IGF-1 circulantes estimulan la liberación de somatostatina que permite inhibir la secreción de GH por las células somatotropas de la hipófisis anterior (64).

El receptor de GH es un receptor de citocina tipo I, es un homodímero activado mediante la unión de la GH y el reclutamiento de residuos de tirosina y la proteína Janus cinasa 2 (JAK2). La principal vía de señalización intracelular de la GH es la vía JAK-STAT (signal transducer and activator of transcription, por sus siglas en inglés). Otras vías de señalización activadas por GH son la MAPK y la fosfatidil inositol 3 cinasa/AKT/mTOR (65).

2.1 Deficiencia de GH y NAFLD

La asociación entre NAFLD y GH se empezó a reconocer en pacientes adultos con hipopituitarismo adquirido. Este grupo de pacientes se caracterizaba por presentar un incremento del tejido adiposo visceral, obesidad, aumento del riesgo cardiovascular y NAFLD a pesar de recibir sustitución hormonal específica para deficiencia de hormonas tiroideas, cortisol u hormonas sexuales. De forma importante, cuando estos pacientes eran tratados con GH restauraban su condición bioquímica, composición corporal y mejoraban la lesión histopatológica del hígado (21). Aunado a este hallazgo, se observó retrospectivamente a una cohorte de pacientes que tenían como antecedente haber cursado con deficiencia de GH desde la edad pediátrica y habían sido tratados únicamente en el período de crecimiento. Después de un tiempo de haber finalizado el tratamiento, los

pacientes con una media de edad de 30 años presentaron un incremento de comorbilidades metabólicas incluyendo la NAFLD hasta en un 29% de los casos (66).

En pacientes con NAFLD se ha documentado la presencia de concentraciones bajas de GH, IGF-1 e IGFBP3, ya sea con esteatosis o con fibrosis, y esta asociación se ha observado aún en pacientes que no tienen deficiencia de GH (67). Se ha reportado también una asociación entre niveles bajos de IGF-1 con el grado de inflamación, degeneración balonizante y el grado de fibrosis en pacientes con NAFLD (68,69). Esta evidencia sugiere que el eje GH - IGF-1 podría desempeñar un papel importante en la fisiología hepática, aún en condiciones de suficiencia hormonal.

El hígado es tan importante como órgano blanco para la GH, que en estudios experimentales realizados en ratones *knockout* para el receptor hepático de GH, la producción de IGF-1 disminuye hasta en un 90%, y las manifestaciones clínicas se traducen en resistencia a insulina, intolerancia a la glucosa, incremento de los ácidos grasos libres y esteatosis hepática severa, lo que en conjunto denota la importancia de la vía de señalización de la GH en el tejido hepático (70,71).

Hasta la fecha, distintos estudios clínicos han demostrado una posible aplicación de GH e IGF-1 en condiciones relacionadas con la obesidad. En estudios observacionales se ha demostrado que la sustitución hormonal con GH tiene efectos muy importantes disminuyendo la adiposidad visceral y la dislipidemia en pacientes adultos con deficiencia de GH, lo cual ha despertado el interés por realizar estudios clínicos administrando esta hormona a pacientes no deficientes de GH pero que cuentan con condiciones clínicas como obesidad visceral y dislipidemia, patologías que después de un año de tratamiento han mejorado, impactando positivamente en otras variables metabólicas como sensibilidad a insulina y menor contenido hepático graso (72).

3. Estrategias experimentales en NAFLD.

El grupo de Nisihizawa y colaboradores en el 2012 observaron en un estudio clínico que existía una asociación entre la deficiencia de GH y la presencia de NAFLD. Con la finalidad de demostrar que la deficiencia de GH causaba NAFLD diseñaron un modelo experimental en ratas enanas naturalmente deficientes de GH, que comparadas con ratas Sprague Dawley demostraron la presencia de NAFLD. En dos condiciones administraron GH e IGF-1 a las ratas deficientes de GH durante 4 semanas, demostrando histopatológicamente que en ambos grupos había una mejoría significativa de la NAFLD (21,22).

Una gran limitante para el conocimiento de la fisiopalogía de la enfermedad y, por lo tanto, para la propuesta de estrategias terapéuticas exitosas, consiste en la dificultad para la simulación de las condiciones histológicas y fisiológicas de la NAFLD en seres humanos. En el ser humano continúa el desafío de poder diagnosticar, estadificar y dar seguimiento al paciente con NAFLD con metodologías diagnósticas no invasivas que sustituyan a la biopsia hepática, que es hasta el momento el imperfecto estándar de oro por el alto riesgo de morbi - mortalidad que conlleva. Ante esta dificultad, se ha recurrido a modelos animales o celulares *in vitro* que mediante distintas estrategias de administración farmacológicas, tóxicas o dietéticas permiten imitar las características patológicas de NAFLD, para de esa forma ampliar el conocimiento fisiopatológico y realizar estudios de tipo farmacológico (73).

3.1 Modelos *in vitro*. Línea celular HepG2.

Dentro de los modelos celulares *in vitro* se encuentra la línea celular HepG2, que es derivada de un hepatoma humano, y dentro de su clase es la línea celular más utilizada en investigación fármaco - toxicológica. Si bien las funciones metabólicas de las células de hepatoma son limitadas comparadas con hepatocitos primarios, estas células ofrecen otras ventajas como su disponibilidad, facilidad en el manejo, una amplia vida útil y un fenotipo estable que no depende de las características del donador. Además, esta línea celular es una línea no tumorigénica pero de alta proliferación (74).

Dentro de las características funcionales de las células HepG2 se encuentran presentes la secreción de proteínas plasmáticas, el metabolismo de colesterol y triglicéridos, el metabolismo y transporte de lipoproteínas, la síntesis de ácidos biliares, la síntesis de glucógeno y la vía de señalización de la insulina. Esta línea celular se ha utilizado de manera constante en estudios de farmacotoxicología como un modelo probado de esteatosis hepática, logrando su caracterización mediante la infiltración de ácidos grasos o en presencia de altas concentraciones de glucosa (74,75).

La asociación entre GH y NAFLD ha sido consistente tanto en estudios clínicos como a nivel experimental, sin que se conozca a fondo qué mecanismos moleculares podrían estar mediando la acción de GH o de su principal efector IGF-1 a nivel hepático al prevenir o modular el desarrollo de NAFLD; este conocimiento podría contribuir a la comprensión de la ya compleja fisiopatología de esta condición hepática y a la incesante búsqueda de dianas terapéuticas específicas.

PLANTEAMIENTO DEL PROBLEMA

La NAFLD es la hepatopatía crónica más frecuente en países occidentales. Este padecimiento está asociado a NASH, cirrosis y hepatocarcinoma celular, patologías con alta morbilidad y mortalidad a nivel mundial, particularmente en la población mexicana. Por su prevalencia creciente y en paralelo a la epidemia de obesidad, la NAFLD representa actualmente uno de los problemas más importantes de salud pública en nuestro país.

Existen diversos enfoques que fortalecen la hipótesis multigolpe que explican parcialmente la compleja fisiopatología de la NAFLD, sin que hasta el momento se pueda tener una idea clara y definitiva de la misma.

En los últimos años se ha reportado una asociación entre la disfunción del eje GH/IGF-1 y la presencia de NAFLD, sin que hasta el momento se conozcan los mecanismos moleculares que median su acción, particularmente en el balance del metabolismo lipídico a nivel hepático.

PREGUNTAS DE INVESTIGACIÓN

1. ¿Puede el tratamiento con GH prevenir o limitar el desarrollo de NAFLD en un modelo hepático *in vitro*?

En caso de haber disminución de NAFLD a la administración de GH:

2. ¿Cuál es la participación de IGF-1 en mediar esta acción?
3. ¿Qué vía metabólica es más importante para el desarrollo de NAFLD, la vía de la lipogénesis *de novo* o la vía de la lipólisis?

JUSTIFICACIÓN

El desarrollo de NAFLD se ha relacionado con múltiples factores de acuerdo a la hipótesis multigolpe, sin que hasta el momento se pueda considerar que existe un conocimiento claro de la patogénesis de la misma. El eje GH/ IGF1 se ha propuesto recientemente como un factor asociado a la patogenia de NAFLD, sin embargo, se desconocen los mecanismos moleculares de su intervención. El estudio de esos mecanismos podría contribuir a un mejor conocimiento fisiopatológico de la enfermedad y a la identificación de posibles dianas terapéuticas.

HIPÓTESIS

Si la GH tiene efectos limitantes en el desarrollo de esteatosis en las células HepG2, entonces las células tratadas con GH mostrarán una reducción en el contenido intracelular de ácidos grasos a dosis crecientes, en comparación con las células HepG2 incubadas en medio de cultivo sin GH.

OBJETIVO GENERAL

Establecer el efecto del tratamiento con GH sobre un modelo celular de esteatosis en células HepG2 cuantificando el contenido intracelular de cuerpos lipídicos y estudiando los mecanismos moleculares potencialmente involucrados en mediar este efecto.

OBJETIVOS ESPECÍFICOS

- Cultivar la línea celular de hepatocitos HepG2 con dosis altas de glucosa para lograr un modelo de esteatosis.
- Cultivar la línea celular de hepatocitos HepG2 con altas concentraciones de glucosa en presencia o ausencia de dosis crecientes de GH recombinante humana.
- Determinar mediante reacción en cadena de la polimerasa en tiempo real con transcriptasa inversa (RT-qPCR, por sus siglas en inglés) la expresión de genes implicados en la vía metabólica de lipogénesis *de novo* en condiciones control y de esteatosis con dosis crecientes de GH.
- Determinar mediante RT-qPCR la expresión de genes implicados en β-oxidación en condiciones control y de esteatosis con dosis crecientes de GH.
- Determinar mediante RT-qPCR la expresión del gen de IGF-1 en condiciones control y de esteatosis con dosis crecientes de GH.
- Determinar mediante Western blot los niveles de las proteínas ChREBP, SREBP1c y FAS implicados en la vía metabólica de lipogénesis *de novo* en condiciones control y de esteatosis con dosis crecientes de GH.
- Determinar mediante Western blot los niveles de proteínas de CPT1A y PPARα implicados en β-oxidación en condiciones control y de esteatosis con dosis crecientes de GH.
- Determinar mediante ensayo por inmunoabsorción ligado a enzimas (ELISA, por sus siglas en inglés) los niveles de IGF-1 en condiciones control y de esteatosis con dosis crecientes de GH.

MATERIAL Y METODOS

1. Células HepG2. Propagación y cultivo.

Utilizamos la línea celular HepG2 de la American Tissue Culture Collection (ATCC®, Manassas, VA) mantenida previamente en criopreservación. Para la propagación y el cultivo empleamos el medio de cultivo de Eagle modificado por Dulbecco (DMEM, por sus siglas en inglés). El DMEM (Thermo Fisher-Scientific, USA) fue adicionado con 10% de suero fetal bovino (SFB), 10nM de buffer HEPES (Sigma-Aldrich, St. Louis, MO) y 50mg/ml de gentamicina (Sigma-Aldrich, St. Louis, MO).

El desarrollo de cultivo se dio en condiciones de 95% de humedad, 37ºC de temperatura y 5% de CO_2 en frasco de cultivo T25 (Corning, USA). (Figura 3).

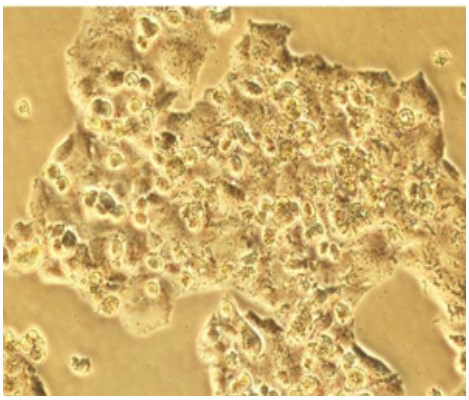

Figura 3. Células HepG2

Al día siguiente del primer cultivo se decantó el medio de cultivo y se reemplazó con medio precalentado con la finalidad de remover células no adheridas y repletar nutrientes. Se vigiló el cultivo bajo microscopio de fases para evaluar las propiedades adhesivas de las células, corroborar la ausencia de contaminación del cultivo, cambios en la morfología celular, el crecimiento en monocapa y la confluencia cada 24hs con recambio de medio de cultivo cada 48hs hasta alcanzar 80% de confluencia (74).

1.1 Subcultivo HepG2

Con la finalidad de subcultivar se desprendieron las células del frasco de cultivo mediante tripsinización. Una vez alcanzado el 80% de confluencia se aspiró el medio de cultivo del frasco se agregó 5ml de tampón fosfato salino (PBS, por sus siglas en inglés) 1X precalentado, mediante la pipeta se hizo un lavado de la superficie de adhesión celular y se esparció gentilmente, se aspiró completamente el PBS y se repitió el proceso una vez más, el lavado de las células tiene la finalidad de remover al 100% del medio de cultivo, ya que

podría interferir con el proceso de tripsinización. Se recuperó al 100% el PBS agregado al frasco.

Se agregó EDTA/tripsina al 0.05% - 2ml (Sigma-Aldrich, St. Louis, MO) precalentado a la superficie y se incubó durante 5 minutos a 37ºC. Al desprenderse las células de la superficie se agregó medio de cultivo precalentado (10ml) a fin de anular la actividad de la tripsina y recuperar las células mediante pipeta fisiológica a un tubo cónico.

Las células se centrifugaron a temperatura ambiente durante 3 minutos a 350g. Se descartó el sobrenadante y se resuspendió el botón celular mediante medio de cultivo precalentado, se pipeteó gentilmente la solución para desagregar los conglomerados celulares. La expansión celular se llevó a cabo a razón de 1:3 en frascos de cultivo T25, repitiendo el proceso hasta llegar al 80% de confluencia (74).

1.2 Caracterización y pruebas funcionales

Durante el quinto pase de la expansión celular y repitiendo el proceso de tripsinización a partir de un frasco de cultivo T25, se obtuvo una muestra de la suspensión celular final para evaluación de la viabilidad celular mediante la exclusión de azul tripano y conteo celular. Empleamos placas de cultivo de 6 pozos (Costar, USA) y sembramos las células HepG2 a una densidad celular de 5 x 10^4 células por cm^2 para los experimentos *in vitro*. Se vigiló el cultivo las primeras 24hs para evaluar adhesión celular y se descartó restos celulares, se realizó recambio de medio del cultivo cada 48hs hasta lograr 70% de confluencia.

1.3 Modelo de esteatosis *in vitro*

Se generó el modelo de estatosis celular *in vitro* a través de altas concentraciones de glucosa en el medio de cultivo. Se eligieron dos condiciones: una control con medio de glucosa 5.5 mmol/L, 1% de SFB y 10nM de buffer HEPES; y una condición de esteatosis en el que al medio de cultivo de las características mencionadas se llevó a una concentración de glucosa 25 mmol/L (76). Se mantuvo el cultivo celular en estas condiciones durante 24hs en las que se logró el modelo de esteatosis (Figura 4).

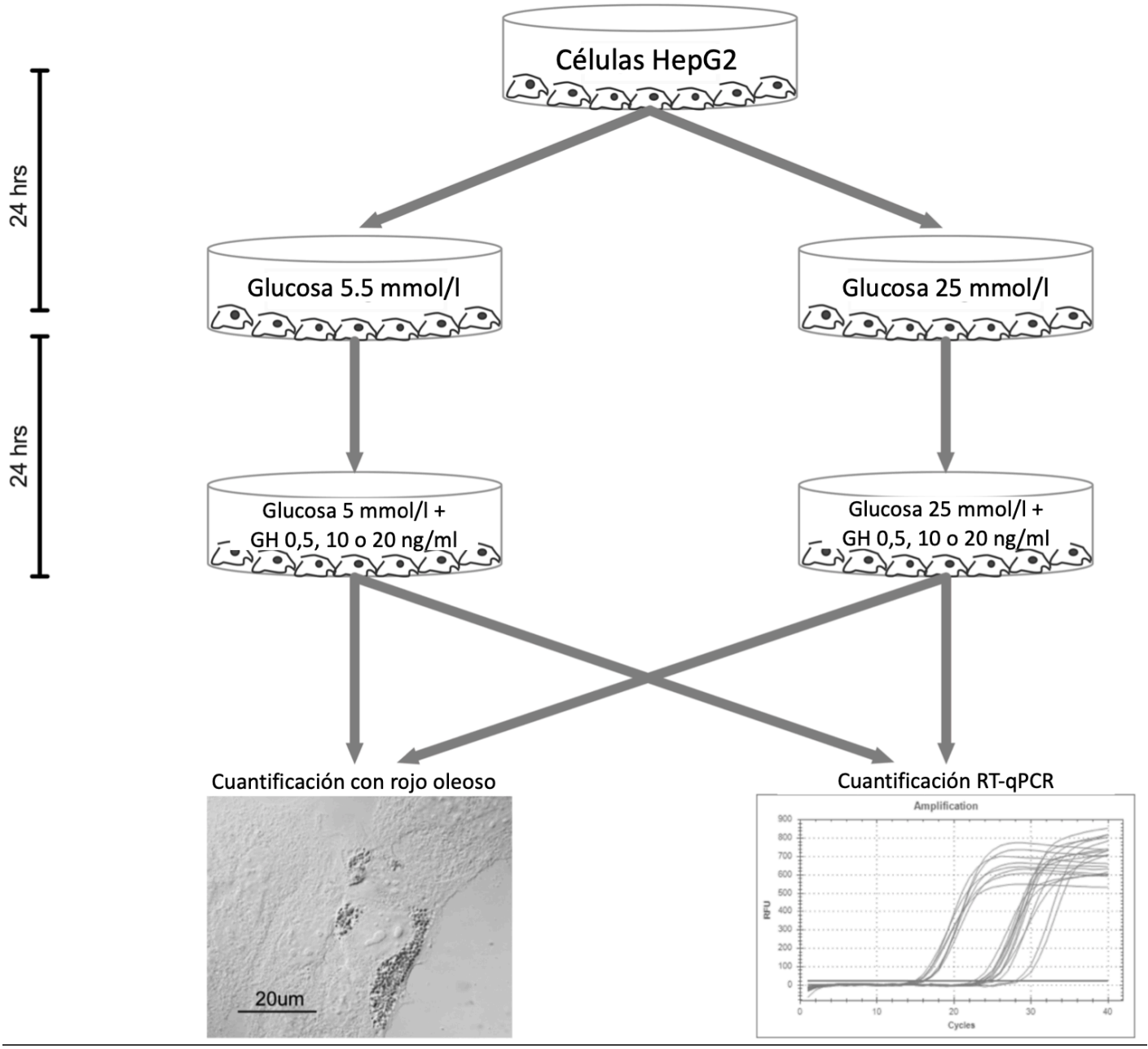

Figura 4. Diseño experimental

2. Tratamiento con hormona de crecimiento (GH)

La hormona de crecimiento recombinante humana (Peprotech, México) se dispuso en 3 dosis: 5, 10 y 20 ng/ml, contrastadas con un control sin GH. Los cultivos celulares control y con esteatosis se sometieron durante 24hs a su respectiva condición de glucosa y se agregó a cada condición las 4 opciones terapéuticas de hormona de crecimiento recombinante

humana (GH, por sus siglas en inglés) durante 24hs más (0, 5, 10 y 20 ng/ml) (Figura 1). Todas las condiciones experimentales antes mencionadas se replicaron por quintuplicado.

3. Tinción con rojo oleoso

Se utilizó la técnica de tinción con rojo oleoso con la finalidad de identificar cualitativamente grasa neutra almacenada en cuerpos lipídicos.

Después de 24hs de administración de GH en sus 4 condiciones al modelo *in vitro* de esteatosis, se retiró el medio de cultivo y se lavó la superficie con PBS 1X en dos ocasiones. Se agregó formaldehído al 3% durante 40 minutos a temperatura ambiente, se lavó con agua bidestilada durante 5 ocasiones, se agregó isopropanolol y se incubó durante 10 minutos, se decantó el isopropanolol y se agregó rojo oleoso al 0.5% en isopropanolol diluida 3:2 con agua destilada durante 15 minutos a temperatura ambiente.

Se lavó el rojo oleoso de la superficie con agua destilada (76,77).

4. Cuantificación histológica de las los cuerpos lipídicos.

Una vez teñidas las células se hizo el conteo de los cuerpos lipídicos por cada 100 células en 10 campos de cada pozo de cultivo. Las microfotografías se tomaron a una ampliación 40X usando un microscopio Nikon Microphot-FXA acoplado a una cámara digital Nikon DXM1200F.

5. Cuantificación del contenido intracelular de triglicéridos.

A partir del cultivo del modelo de esteatosis expuesto a la administración de GH a 0, 5, 10 y 20 ng/ml, se aspiró el medio de cultivo y se lavó la superficie de cultivo con PBS 1X hasta en 2 ocasiones. Se administró 250 µl de lipasa (Sigma-Aldrich, St. Louis, MO) durante 4 minutos. Una vez detenida la digestión enzimática, se cuantificó la concentración proteica mediante absorciometría a 595 nm utilizando el Bradford Protein Assay (Bio-Rad, USA).

La concentración de triglicéridos se midió incubando la suspensión celular en presencia de 0.4 ml de metanol/0.5 ml de cloroformo durante 45 minutos. Se agregó 0.8 ml cloruro de sodio 100 mM, se desecó la fracción de triglicéridos a 45 ºC por 30 minutos.

Se agregó 50 µl de solución estándar de triglicéridos (Abcam, Cambridge, UK) a la fracción de triglicéridos y se cuantificó la concentración de los mismos por espectrometría a 570 nm utilizando un lector de microplacas (76).

6. Aislamiento de RNA total

Las células HepG2 cultivadas en presencia de 0, 5, 10 o 20 ng/ml de GH durante 24hs, una vez despegadas de la superficie mediante la solución tripsina-EDTA al 0.05% (Sigma-Aldrich, St. Louis, MO) se centrifugaron a 200 g/ 8 ºC y se agregó TRIzol (Invitrogen, Carlsbad, CA) a 4 ºC durante 1 minuto. Se agregó 0.2ml de cloroformo frío (Sigma-Aldrich, St. Louis, MO) por cada mililitro de suspensión celular y se incubó a 4 ºC durante 15 minutos. Se centrifugó la muestra a 20800 g /4 ºC durante 15 minutos, después de lo cual se recuperó la fase acuosa y se almacenó en tubos Eppendorf de 1.6ml (Axygen, Mexico).

El RNA total se precipitó con 0.8ml de alcohol isopropílico (J.T. Baker, Mexico) a -20ºC por 2 horas. Las muestras de RNA se centrifugaron a 20800 g/4ºC por 15 minutos y se descartó el sobrenadante. Posteriormente, el botón de RNA se enjuagó con etanol absoluto al 100% (J.T. Baker, Mexico) en 3 ocasiones y se resuspendió en agua libre de RNAasas con DEPC (Sigma-Aldrich, St. Louis, MO). La concentración total de RNA se midió por absorbancia a 260 / 280 nm y se verificó la integridad del RNA mediante electroforesis sobre gel de agarosa desnaturalizante al 1.0 % (Promega, Uniparts, Mexico) en presencia de 2.2 mmol/L de p-formaldehído.

7. Expresión de ChREBP, SREBP1c, FAS, CPT1A, PPAR-α e IGF-1 por RT-PCR en tiempo real.

Las muestras de RNA de las células HepG2 fueron sujetas a un proceso de transcripción reversa utilizando un sistema de retrotranscripción M-MLV y Oligo(dT) primer (Invitrogen, Carlsbad, CA). El ácido desoxirrebonucleico complementario (cDNA, por sus siglas en inglés) se amplificó por reacción en cadena de la polimerasa cuantitativa (qPCR, por sus siglas en inglés) empleando –oligonucleótidos específicos para humano con la

finalidad de detectar la expresión del ácido ribonucleico mensajero (mRNA) de ChREBP, SREBP1c, FAS, CPT1A, PPAR-α e IGF-1 (Tabla 1).

Las muestras de cDNA (100 ng/ml) fueron colocadas de manera individual en el termociclador de tiempo real BIO-RAD CFX96 en presencia de un Supermix de PCR, consistente en 7.5 ml de SsoAdvanced Universal SYBR Green (BIO-RAD, USA), 0.6 ml de solución primer-específica, 5.3 ml de agua, y 1 ml de muestra de DNA por tubo. Se utilizó el oligonucleótido de RNA de GAPDH como gen endógeno para el cálculo y normalización por doble delta CT (DDCT), los resultados se expresaron en veces de cambio.

Tabla 1. Secuencias de los oligonucleótidos para amplificación de RT-qPCR

Gen	Iniciador directo	Iniciador reverso	Longitud (pb)
IGF-1	5'-TGGTGGACGCTCTTCAGTTC -3'	5'-TCCGGAAGCAACACTCATCC -3'	123
ChREBP	5'- CCGTCCAGACAGCAACAAGA -3'	5'- GCTGCTGGCACAGGTTAATG -3'	275
FAS	5'- TGCGTGGCCTTTGAAATGTG -3'	5'- CTCCATGTCCGTGAACTGCT - 3'	260
SREBP1c	5'- GATGCGGAGAAGCTGCCTAT -3'	5'- GCTGTGTTGCAGAAAGCGAA -3'	234
CPT1A	5'- GACGGCTATGGTGTGTCGTA -3'	5'- TCCAGCCCAGCACATGAAC -3'	202
PPAR-α	5'- ACACCGAGGACTCTTGCGA -3'	5'- GGAAAGGGCAAGTCCCGATG -3'	207
GAPDH	5'- AAAGCCTGCCGGTGACTAAC -3'	5'- TTCCCGTTCTCAGCCTTGAC -3'	300

pb: pares de bases.

8. Medición de la concentración de IGF-1 por ELISA

El sobrenadante de las distintas condiciones de cultivo se recolectó y almacenó en tubos Eppendorf de 2.0 ml, libre de pirógenos (Axygen, México) a -80ºC hasta su evaluación. Los niveles de IGF-1 se cuantificaron mediante la técnica de ELISA de acuerdo a las instrucciones del fabricante (Sigma-Aldrich, St. Louis, MO).

9. Evaluación de la concentración de las proteínas: ChREBP, SREBP1c, FAS, CPT1A y PPAR-a por Western blot.

Las células HepG2 de las distintas condiciones de cultivo se sometieron a la administración de un buffer de extracción de proteínas con 500 mmol&l de Tris-Hcl (1 ml/ 50 x 10^4 células) y un coctel inhibidor de proteasas (Calbiochen, Germany). Se recuperó el sobrenadante de la suspensión celular después de la centrifugación a 20800 g/8ºC durante 15 minutos. La concentración proteica se cuantificó por absorbancia a 595 nm mediante el método de Bradford (Bio-Rad, USA). Se calentó 30 µg de proteína total en un sistema de taponamiento de Laemmli y la separación se realizó mediante electroforesis en gel de poliacrilamida con dodecilsulfato sódico (SDS-PAGE) con 10% de acrilamida. Después, las proteínas se transfirieron a membranas de difluoruro de polivinilideno (PVDF) (Thermo Fisher Scientific, Inc., USA), estas se bloquearon con PBS 1x (0.2% de polisorbato 20) conteniendo 1% de SFB durante 12 hs. Después de hasta 5 lavados con PBS 1X - polisorbato 20, las membranas PVDF se incubaron en presencia de anti ChREBP humano de conejo 1:500 (95KDa), anti SREBP1c humano de ratón 1:100 (132KDa), anti FAS humana de ratón 1:100 (273 KDa), anti CPT1A humano de ratón 1:250 (88KDa), o anti PPAR alpha humano de ratón 1:150 (52KDa) (Abcam, Cambridge, UK) durante 60 minutos a temperatura ambiente. Se utilizó la reacción de peroxidasa-diaminobencidina (Sigma-Aldrich, Mexico) para visualizar las bandas de proteína que posteriormente fueron cuantificados mediante densidad óptica usando β-actina como proteína control.

10. Análisis estadístico

Los datos se obtuvieron en quintuplicados experimentales independientes. Se utilizó el test de Shapiro-Wilk para evaluar la normalidad de los datos. Los datos se analizaron por medio de análisis de varianza (ANOVA) de un vía, seguida de un análisis post hoc mediante Games-Howell para estimar las diferencias significativas de los niveles de expresión de RNA mensajero y de proteínas de ChREBP, SREBP1c, FAS, CPT1A, PPAR-α e IGF-1, así como la cuantificación de los cuerpos lipídicos entre las células HepG2 control y células con esteatosis tratadas durante 24hs con las diferentes concentraciones de GH.

Los datos se expresaron en medias ± desviaciones estándar, se consideraron diferencias significativas con un valor de $p < 0.05$. Los resultados se analizaron y graficaron utilizando

el software GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA 92037 USA) e IBM SPSS Statistics version 26.0 (IBM, Armonk, NY, USA).

RESULTADOS

El diseño experimental que se utilizó para generar el modelo de esteatosis *in vitro* se muestra en la figura 4.

En condiciones normales de glucosa (5.5 mmol/L), las células HepG2 sin exposición a GH (modelo control) muestran una discreta señal positiva a la administración de la tinción de rojo oleoso (Figura 5A). A la administración de GH en una concentración de 5 ng/ml, se muestra una menor presencia de cuerpos lipídicos en comparación con las células control sin GH (Figura 5B). A la administración de GH en las concentraciones de 10 y 20 ng/ml, en condiciones control no hubo diferencia comparada con la condición control sin tratamiento con GH (Figura 5C y 5D).

Con respecto a las células HepG2 cultivadas en condiciones de altas dosis de glucosa (25 mmol/l) que corresponden al modelo *in vitro* de esteatosis, en ausencia de GH podemos observar un incremento importante en la tinción positiva para rojo oleoso que contrasta de manera significativa con el modelo control sin GH (Figura 5A), lo cual nos indica la presencia de una gran cantidad de cuerpos lipídicos (Figura 5E).

A la administración de 5 ng/ml de GH en el modelo de esteatosis (glucosa 25mmol/l), se observa claramente una reducción de cuerpos lipídicos (Figura 5F), y observamos una disminución aún mayor de la señal de rojo oleoso a la administración de GH en las concentraciones de 10 y 20 ng/ml (Figura 5G y 5H, respectivamente).

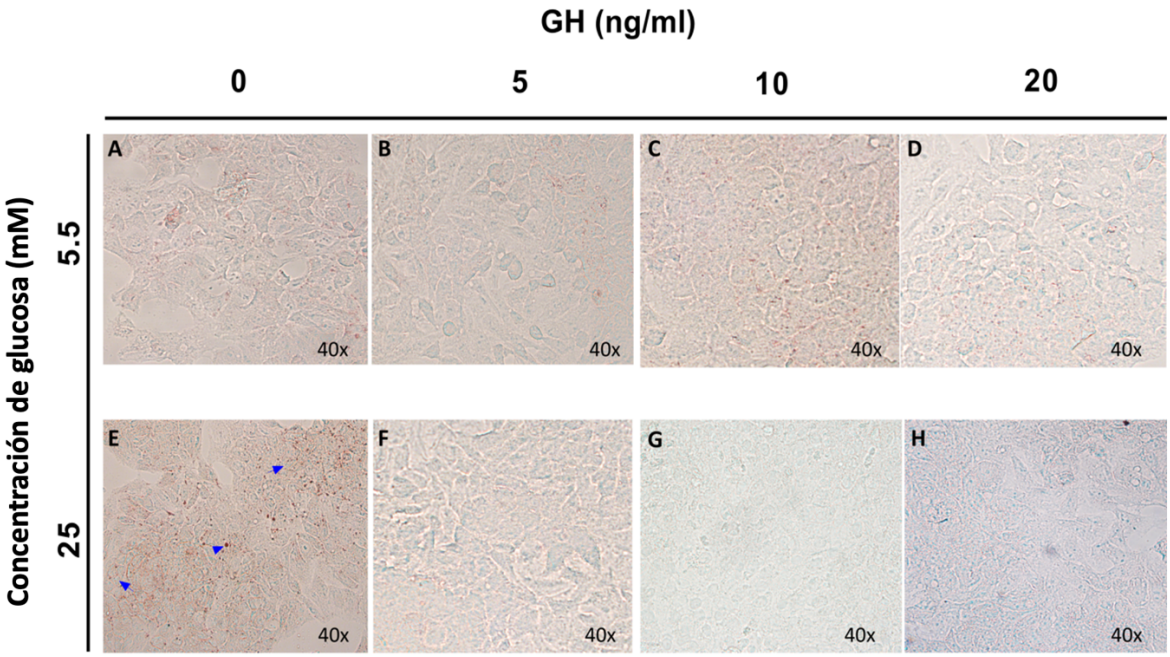

Figura 5. Microfotografías de células HepG2 cultivas en condiciones control (5.5 mmol/L de glucosa) y en condiciones de esteatosis (25 mmol/L de glucosa) tratadas con dosis crecientes de GH. **A**-Condición control (5.5 mmol/l de glucosa) sin esteatosis y sin GH. **B**-Condición control de glucosa con GH 5ng/ml. **C**-Condición control de glucosa con GH a 10ng/ml. **D**- Condición control de glucosa con GH a 20ng/ml. **E**- Condición de esteatosis (25 mmol/l de glucosa) sin GH. **F**-Condición de esteatosis (25 mmol/l de glucosa) con GH a 5ng/ml. **G**- Condición de esteatosis (25 mmol/l de glucosa) con GH a 10ng/ml. **H**- Condición de esteatosis (25 mmol/l de glucosa) con GH a 20ng/ml.
En comparación con el panel A (esteatosis sin GH), en las condiciones F,G y H se observa una reducción importante de las cuerpos lipídicos destacados mediante la tinción de rojo oleoso (señalados por flechas azules). Microfotografías amplificadas a 40X con microscopio Nikon Micophot-FXA acoplado a cámara digital Nikon DXM1200F. (n= 5)

La disminución aparente de la acumulación lipídica en los hepatocitos HepG2 cultivados en presencia de GH apreciada en las microfotografías se corroboró mediante la cuantificación del número de cuerpos lipídicos por cada 100 células y la cuantificación del contenido intracelular de triglicéridos.

Respecto a los hepatocitos HepG2 cultivados en condiciones control, sin la presencia de GH se identifica una media de 14.81 ± 12.35 cuerpos lipídicos por cada 100 células (Figura 6A, conjunto de columnas en color blanco). En las mismas condiciones de cultivo (5.5 mmol/l de glucosa), pero con exposición a 5, 10 ó 20 ng/ml de GH se cuantifican 8.33 ± 14.64; 17.21 ± 14.41; y 11.56 ± 10.88 cuerpos lipídicos por cada 100 células, respectivamente. (Figura 6A, conjunto de columnas en color blanco).

En el caso de los hepatocitos HepG2 cultivados en condiciones de esteatosis (25 mmol/l de glucosa) y sin tratamiento con GH, observamos un incremento al menos dos veces mayor en la cuantificación de cuerpos lipídicos (29.07 ± 23.09 por cada 100 células) comparado con la condición control (Figura 6A, panel en color negro). En el modelo de esteatosis al administrar dosis crecientes de GH a 5, 10 y 20 ng/ml se observa una reducción de 2.3, 7.6 y 4 veces en el número de cuerpos lipídicos, respectivamente, comparadas con el modelo de esteatosis sin tratamiento con GH (Figura 6A, conjunto de columnas color negro).

La cuantificación del contenido intracelular de triglicéridos nos da objetividad y confirma el efecto del tratamiento con GH en el modelo de esteatosis con células HepG2. Tal como se podría esperar, en el caso de las células HepG2 en condiciones control, no existe una diferencia significativa en el contenido de triglicéridos independientemente de la administración o no de GH (Figura 6B, conjunto de columnas en color blanco).

De manera consistente con los hallazgos histológicos, la cuantificación del contenido de triglicéridos en la condición de esteatosis (25 mmol/l de glucosa) y en ausencia de GH se incrementa de manera significativa, así mismo podemos observar que la administración de GH a dosis crecientes disminuye significativamente también el contenido de triglicéridos (Figura 6B, conjunto de columnas en color negro).

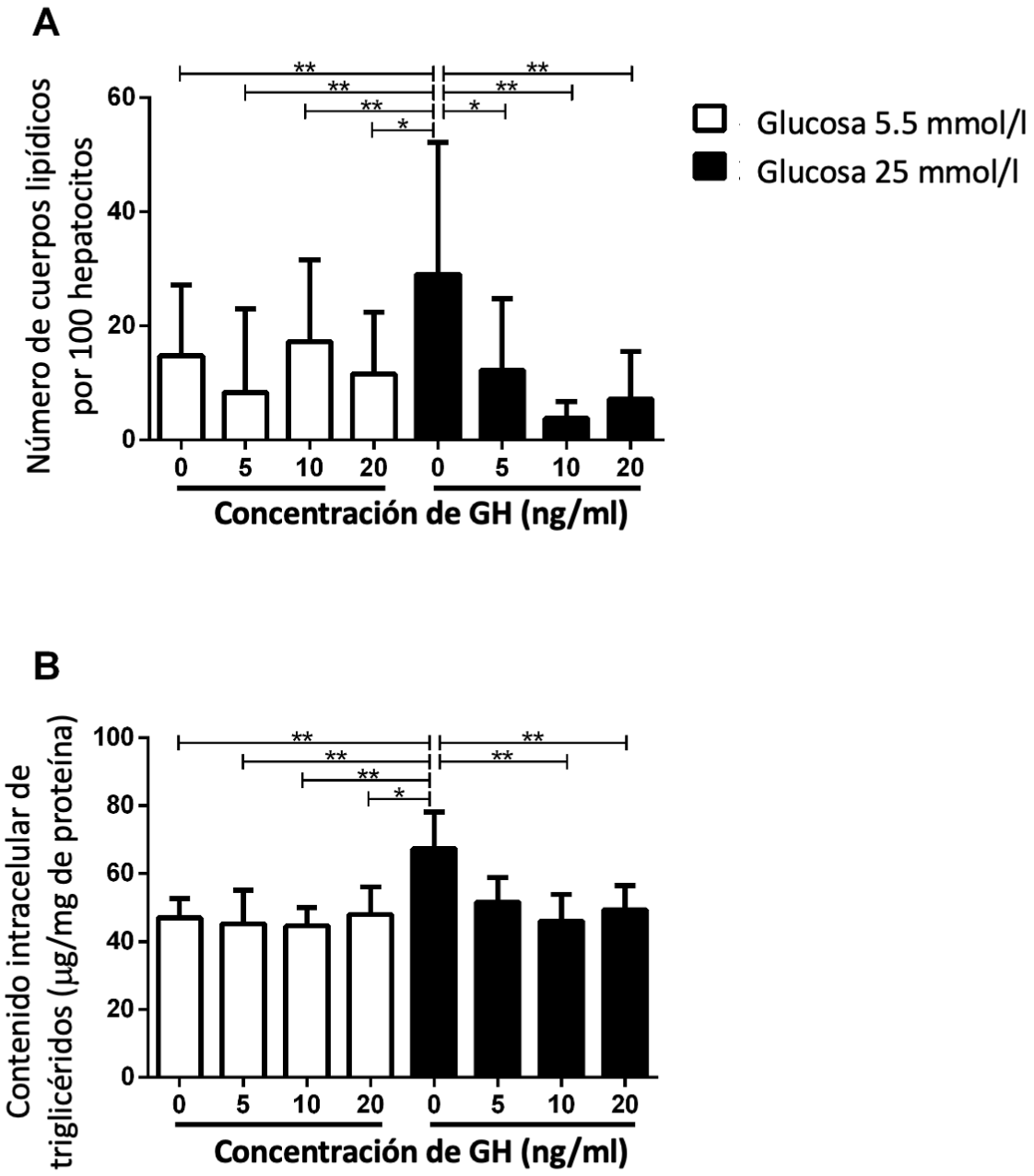

Figura 6. **A**.- Cuantificación del número de cuerpos lipídicos por cada 100 células en células HepG2 cultivadas en condición control (5.5 mmol/l de glucosa) y en células HepG2 cultivadas en condiciones de esteatosis (25 mmol/l de glucosa) a la administración de dosis crecientes de GH 0, 5, 10 y 20 ng/ml. **B**.- Cuantificación del contenido de triglicéridos en las condiciones antes mencionadas. Conjunto de columnas en color blanco muestra la condición control, conjunto de columnas en color negro muestra la condición de esteatosis. (n= 5; $p < 0.05$).

Se sabe que IGF-1 funge como el principar efector mitogénico y metabólico de las acciones de GH en los mamíferos. En un intento de explicar el posible mecanismo molecular por el cual la GH disminuye la esteatosis en células HepG2, se cuantificó la expresión de IGF-1 en las distintas condiciones experimentales. Observamos que en la condición control, la administración de GH a 10 ng/ml incrementa hasta 1.7 veces la expresión comparada con la condición con GH a 5 ng/ml (Figura 7A, conjunto de columnas en color blanco), y en el caso de la administración de GH a 20 ng/ml pareciera existir una retroalimentación negativa a una posible elevación de la concentración de IGF-1 proteica.

Además, observamos que en el modelo de esteatosis (glucosa 25 mmol/l), independiente de la administración de cualquier concentración de GH no existe un incremento en la expresión de los niveles de IGF-1 (Figura 7A, conjunto de columnas en color negro), contrastando concretamente a la respuesta estimuladora que se da a la administración de GH a 10 ng/ml en una condición control (glucosa 5 mmol/l) (Figura 7A, conjunto de columnas en color negro). La síntesis de proteína IGF-1 sigue el mismo comportamiento observado en la expresión del RNA mensajero, corroborando que no hay síntesis proteíca en condiciones de hiperglucemia (Figura 7B, conjunto de columnas en color negro).

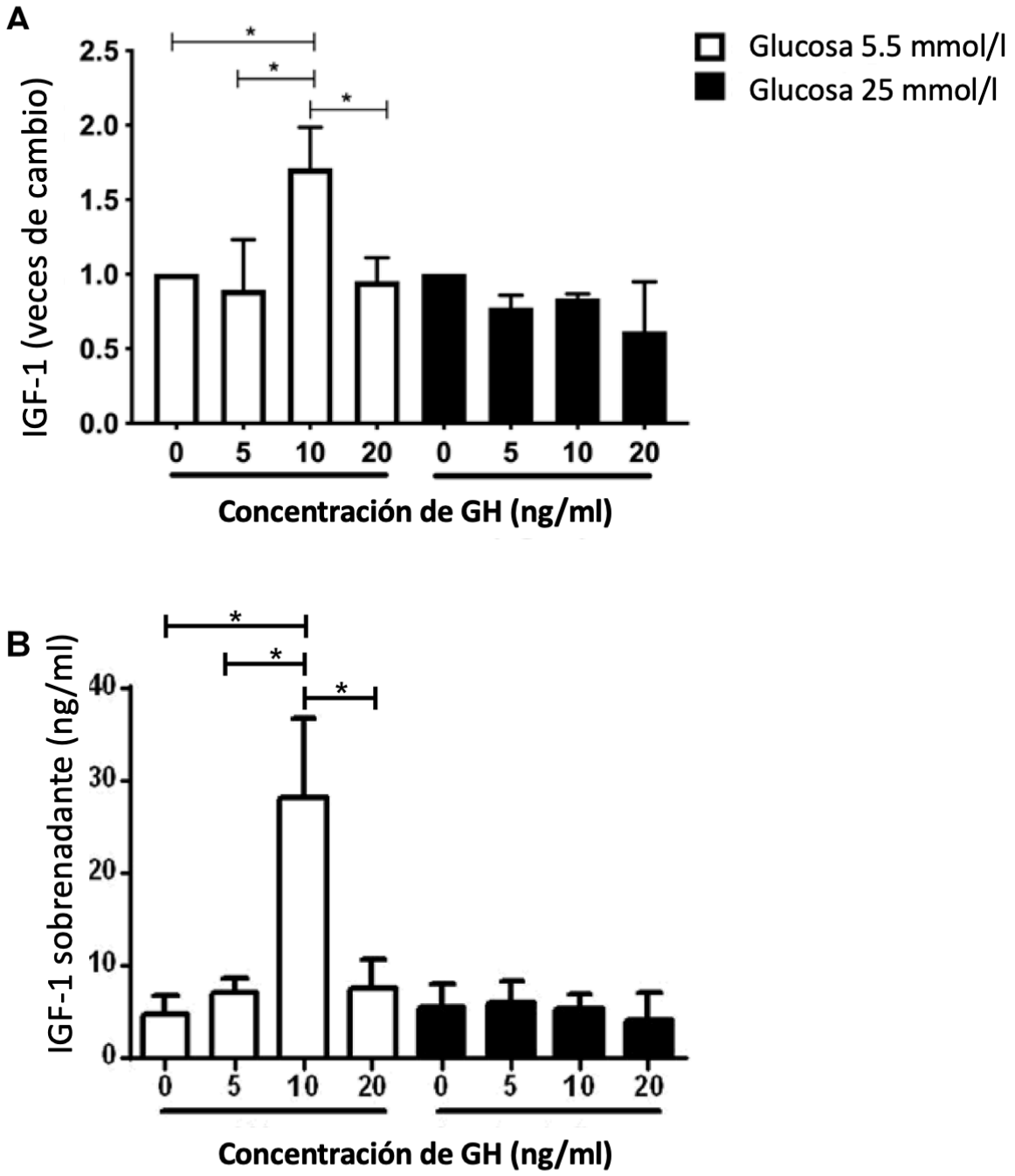

Figura 7. A- Niveles relativos de expresión de IGF-1 según la condición control (5.5mmol/l de glucosa) o de esteatosis (25 mmol/l de glucosa) en células HepG2 tratadas con dosis crecientes de GH. B- Niveles de proteína IGF-1. (n= 5; $p < 0.05$).

A fin de dar respuesta a los posibles mecanismos en los que podría influir la modulación de la esteatosis a la GH se planteó explorar dos principales vías metabólicas involucradas en la acumulación lipídica del tejido hepático: la DNL y la β-oxidación. Para explorar DNL se

evalúo la expresión de ChREBP, SREBP1c y de FAS. Para β-oxidación se evalúo la expresión de CPT1A y PPAR-α.

La expresión del gen de ChREBP se da habitualmente tanto en músculo esquelético como en tejido hepático y su activación es dependiente de la concentración de glucosa. Lo que observamos en nuestra condición control es que no hay expresión de ChREBP independientemente de la administración de GH en las distintas concentraciones (Figura 8A, conjunto de columnas en color blanco). Acorde a lo esperado, en el modelo de esteatosis (25 mmol/l de glucosa) sin GH, ChREBP incrementó hasta 3.5 veces más su expresión (Figura 8A, conjunto de columnas en color negro), a la administración de GH se observa una disminución dosis-dependiente, siendo más relevante la disminución a la administración de GH a 10 ng/ml, demostrando una diferencia estadísticamente significativa con la condición de esteatosis sin GH y con la condición de esteatosis con GH a 5 ng/ml (Figura 8A, conjunto de columnas en color negro).

SREBP1c es un gen involucrado también en la vía de DNL, tiene una activación indirecta, ya que en presencia de glucosa incrementan los niveles de insulina y justo es esta última hormona la responsable de activar en el tejido hepático una mayor expresión de SREBP1c. En nuestro diseño experimental no se demuestra una diferencia de expresión estadísticamente significativa en la condición control (Figura 8B, panel conjunto de columnas en color blanco). En la condición de esteatosis no pudimos comprobar una mayor expresión de SREBP1c ni en la condición sin GH, ni a las distintas concentraciones de GH (Figura 8B, conjunto de columnas en color negro).

La expresión del gen FAS se considera un paso determinante en la lipogénesis tanto en tejido adiposo como en tejido hepático. En nuestro trabajo se muestra como en la condición control (5.5 mmol/l de glucosa) hay una baja expresión del gen FAS, aunque con un incremento significativo a la administración de GH a 5 ng/ml (Figura 8C, conjunto de columnas en color blanco). De acuerdo a lo pronosticado, en la condición de esteatosis sin GH, el incremento en la expresión de FAS es hasta 17 veces mayor comparado con la condición control y notamos también como la administración de GH a 5, 10 y 20 ng/ml

prácticamente nulifica la expresión de este gen (Figura 8C, conjunto de columnas en color negro).

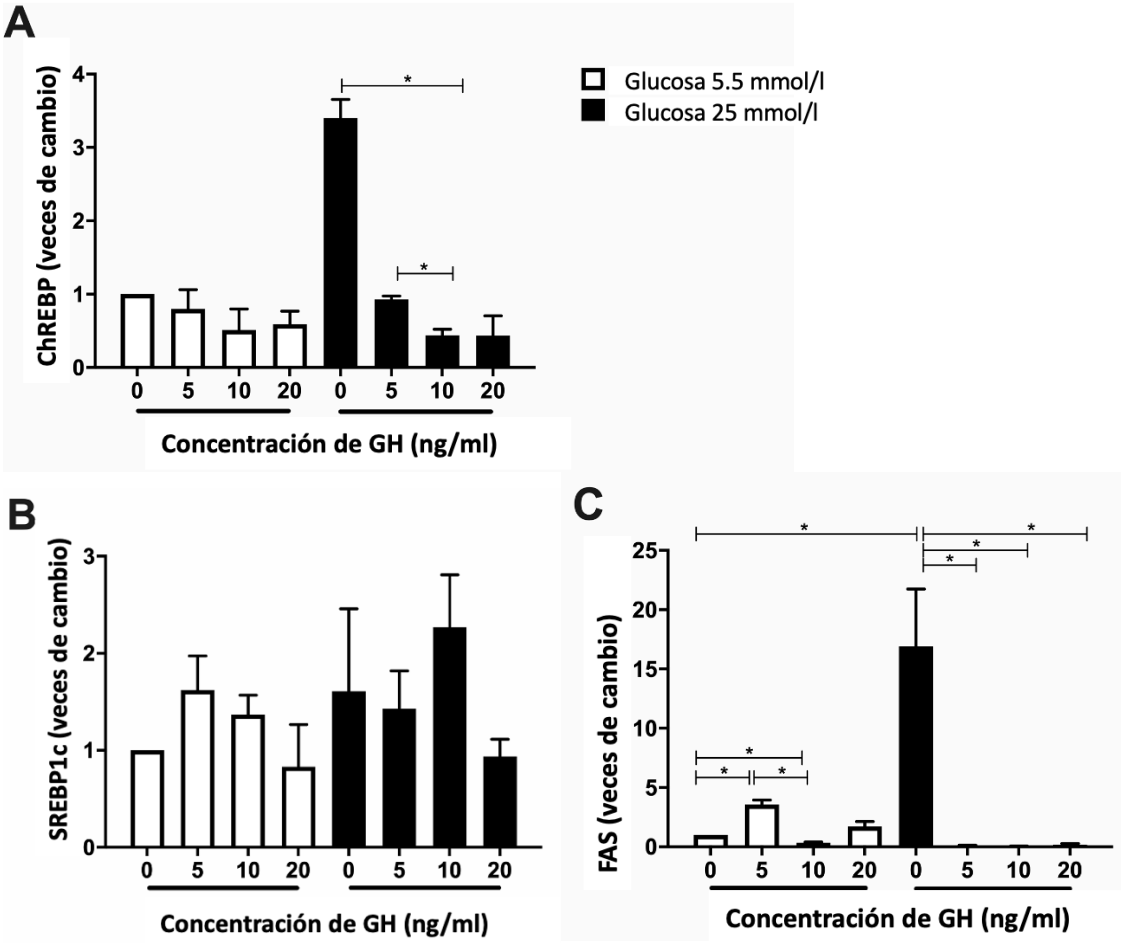

Figura 8. Expresión de genes de lipogénesis en el modelo control y en el de esteatosis en presencia de GH 0, 5, 10 y 20 ng/ml. A - ChREBP, muestra hasta 3 veces mayor expresión de la condición de esteatosis (25mmol/l de glucosa) respecto a la condición sin esteatosis (5.5mmol/l), con disminución estadísticamente significativa a la administración de GH a 10ng/ml en condición de esteatosis. B - SREBP1c, no se observa diferencias de expresión entre condición control y esteatosis. C - FAS, expresión hasta 17 veces mayor en la condición de esteatosis sin GH respecto a la condición control, se observa disminución estadísticamene significativa a la administración de 5, 10 y 20ng/ml de GH en la misma condición. (n= 5; $p < 0.05$).

La expresión del gen CPT1A es primordial en el proceso de β-oxidación de los ácidos grasos. Nuestros resultados en la condición control parecieran mostrar una tendencia a incrementar la expresión de CPT1A cuando se administra GH en las diferentes concentraciones, sin embargo, no logramos encontrar una diferencia estadísticamente significativa (Figura 9A, conjunto de columnas en color blanco). Así mismo, no encontramos diferencias estadísticamente significativas en la expresión de CPT1A en las

condiciones de esteatosis, independientemente de la administración de las distintas concentraciones de GH (Figura 9A, conjunto de columnas en color negro).

El gen de PPAR-α es un mediador esencial del proceso lipolítico, sin embargo, ni en la condición control, ni en la de esteatosis pudimos observar una diferencia estadísticamente significativa, independientemente de las concentraciones de GH administradas (Figura 9B).

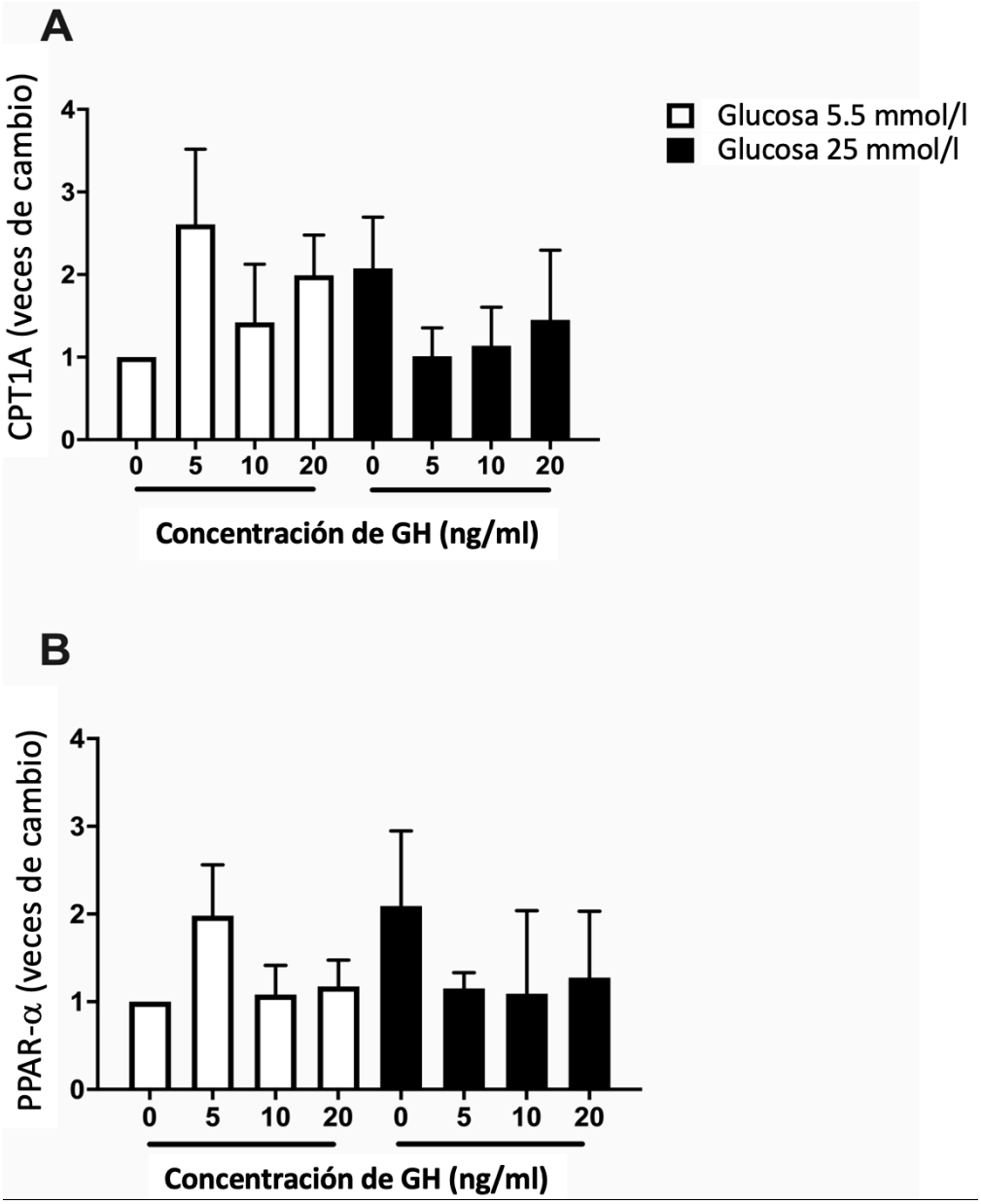

Figura 9. Expresión de genes de β-oxidación en el modelo control y en el de esteatosis en presencia de GH 0, 5, 10 y 20 ng/ml. A - CPT1A. B - PPAR-α. (n= 5; $p < 0.05$).

En la figura 10A se puede apreciar la síntesis de las proteínas correspondientes a las vías de lipogénesis y lipólisis descritas con anterioridad. Las bandas del gel de poliacrilamida muestran de forma cualitativa la síntesis de las proteínas ChREBP, SREBP1c, FAS, CPT1A, y PPAR-α, cultivados en condiciones control y de esteatosis en presencia de dosis crecientes de GH. En este panel observamos una mayor síntesis de ChREBP y FAS en la condición de esteatosis, así como la disminución progresiva de ambas proteínas a dosis crecientes de GH (Figura 10A).

En cuanto a la síntesis de la proteína ChREBP, podemos observar que en la condición control no hay cambios en la concentración de la misma independientemente de la concentración de GH (Figura 10B, conjunto de columnas en color blanco), mientras que en la condición de esteatosis incrementa hasta 2 veces la concentración respecto a la condición control, la cual disminuye progresivamente a la administración de dosis crecientes de GH, con un máximo efecto a la administración de 20 ng/ml de GH (Figura 10B, conjunto de columnas en color negro).

En concordancia con la expresión de RNA mensajero, SREBP1c no demostró una mayor síntesis de la proteína en la condición de esteatosis respecto a la control independientemente de la presencia o no de GH (Figura 10C).

Respecto a la síntesis de FAS, podemos observar que no existe un cambio en la concentración de la misma en la condición control independientemente de la dosis de GH administrada (Figura 10D, conjunto de columnas en color blanco), mientras que en la condición de esteatosis podemos observar hasta 4 veces mayor concentración respecto a la condición control. De forma interesante, podemos observar que en condiciones de esteatosis la administración creciente de 5, 10 o 20 ng/ml de GH suprime de manera

significativa la síntesis de FAS, de la misma forma en que se observó la disminución en la expresión génica de FAS (Figura 10D, conjunto de columnas en color negro).

La síntesis de CPT1A o PPAR-α se comportó de la misma forma que su expresión génica y no demostró cambios en las condiciones control o de esteatosis, independientemente de la dosis de GH administrada (Figura 10E y 10F, respectivamente).

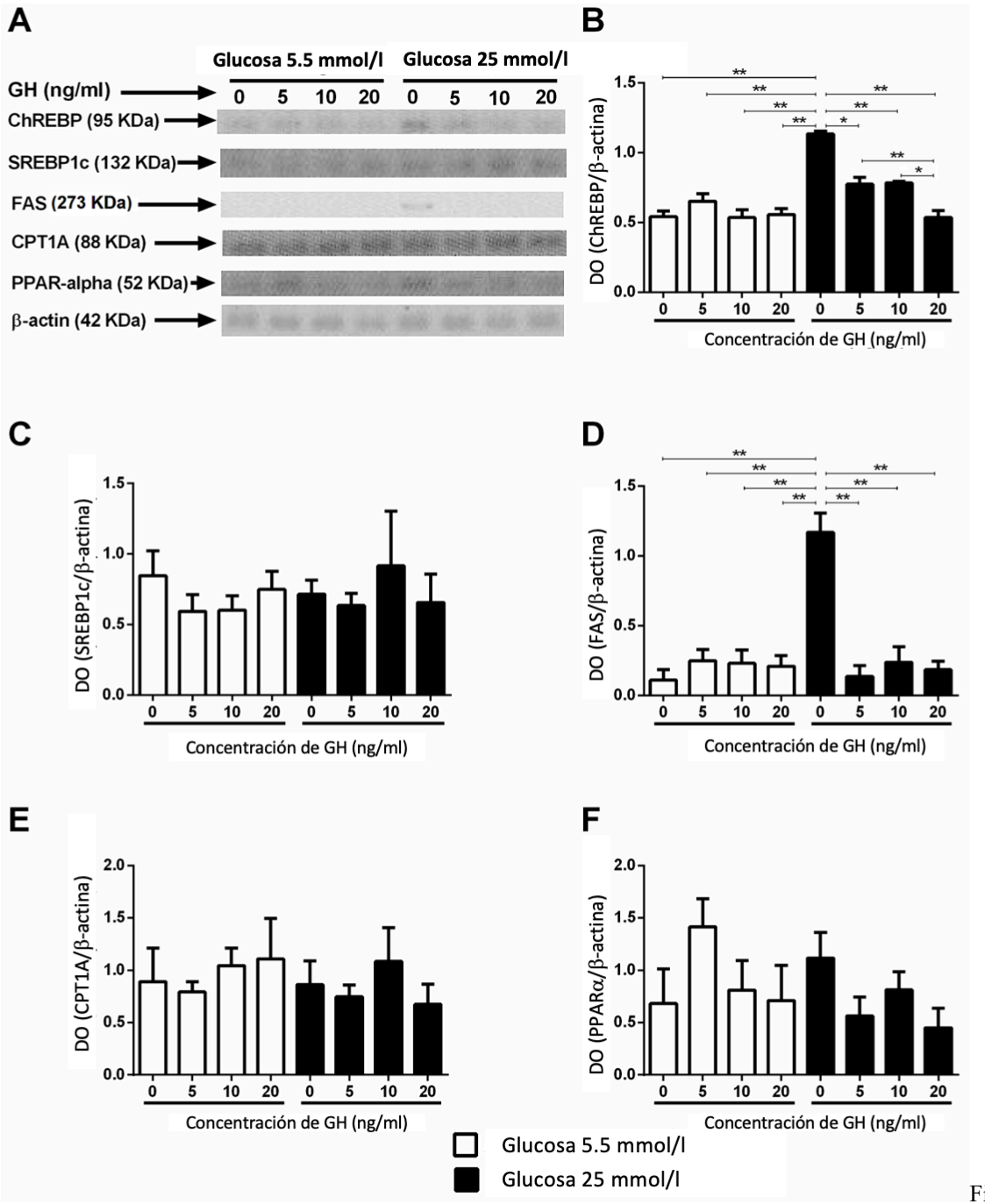

Figura 10. Abundancia de proteína de ChREBP, SREBP1c, FAS, CPT1A and PPAR-α en células HepG2 cultivadas en condiciones control (glucosa 5.5 mmol/l) y de esteatosis (glucosa 25 mmol/l) en condiciones de GH: 0, 5, 10 y 20 ng/ml. A - Gel de poliacrilamida que muestra las bandas correspondientes a las proteínas (ChREBP, SREBP1c, FAS, CPT1A, y PPAR-α de las condiciones experimentales. B - ChREBP. C - SREBP1c. D - FAS. E - CPT1A. F - PPAR-α. Se muestran diferencias estadísticamente sifnificativas en los paneles B y D correspondientes a ChREBP y FAS respectivamente, destaca en ambos casos una concentración más alta en la condición de esteatosis sin GH comparada con el control (5.5 mmol/l glucosa) y una disminución estadísticamente significativa en condiciones de esteatosis a la administración de GH a 5, 10 y 20ng/ml, encontrando una mayor disminución en la concentración de FAS. (n= 5; $p < 0.05$).

DISCUSIÓN

En la literatura se ha documentado una asociación entre la deficiencia de GH y la presencia de NAFLD, tanto en estudios clínicos como en modelos animales (21,22). Sin embargo, todavía se desconoce el mecanismo que pudiera estar mediando esta asociación. Una limitante para atribuir una causalidad o una interacción directa es que tanto seres humanos como animales son sistemas complejos que a la par de una disfunción del eje GH - IGF-1 pueden cursar simultáneamente con otras alteraciones endocrinas y nutricionales, alteraciones en el metabolismo de lípidos y carbohidratos, que por sí mismos podrían contribuir al desarrollo de NAFLD (31,63).

En este trabajo utilizamos un modelo de esteatosis hepática *in vitro* mediante células HepG2 y la presencia de altas concentraciones de glucosa (25 mmol/l), modelo al cual se administró GH en distintas concentraciones, con la finalidad de probar que había una disminución de la NAFLD ante la presencia de GH y de evaluar dos posibles mecanismos involucrados en la acumulación intrahepática de ácidos grasos: lipogénesis y β-oxidación; en ambos casos elegimos genes esenciales de ambos procesos y de esa manera poder evaluar la participación de ambas vías metabólicas.

Las concentraciones de GH que utilizamos en este trabajo, se propusieron a partir del conocimiento previo de los niveles de GH medidos ante una respuesta estimulada en seres humanos. En el contexto de deficiencia relativa en seres humanos con obesidad se han reportado valores promedio de 5 ng/ml, y los valores en los que se considera suficiencia de GH corresponden a 10 ng/ml (78–80). La dosis de 20 ng/ml se administró para evaluar los efectos de la administración suprafisiológica de GH.

Nuestro trabajo demuestra que la GH disminuye de forma directa la infiltración lipídica en un modelo de esteatosis *in vitro* y de manera independiente a otros mecanismos que pudieran actuar como confusores en un sistema vivo. Este hallazgo se suma a la evidencia previamente reportada en estudios clínicos y experimentales en modelos animales y refuerza un papel causal de la ausencia de GH como inductor de esteatosis (21,22,81).

Este trabajo también se propuso dilucidar los posibles mecanismos involucrados en la modulación de la infiltración grasa en tejido hepático por la administración de GH. Como sabemos, IGF-1 es el principal efector de los mecanismos metabólicos y mitogénicos de la GH (71,80,82). La expresión positiva de IGF-1 en nuestro trabajo se dio únicamente en la condición control a la administración de 10 ng/ml de GH, lo cual pudimos demostrar también al evaluar la síntesis proteica de IGF-1. Si bien es cierto que los modelos celulares no son extrapolables a las condiciones *in vivo*, este hallazgo podría tener su similitud en el contexto clínico de una persona sana con niveles de GH en rango de suficiencia (83). En el caso de la condición control con GH a 5 ng/ml podemos observar que no hay un incremento en los niveles de IGF-1, lo que pudiera asemejarse en el contexto clínico a que la concentración mencionada se clasifica como deficiencia relativa de GH (84). Observamos que a la administración de GH a 20 ng/ml existe una disminución en la expresión de IGF-1 así como en su síntesis proteica comparada con la respuesta con 10 ng/ml. Esta observación muy probablemente pueda atribuirse a una retroalimentación negativa en la vía de señalización Jak-STAT y la activación de proteínas supresoras de la vía de señalización de citocinas (SOCS, por sus siglas en inglés), acciones que impactarían desensibilizando o generando una ubiquitinación del receptor de GH (85).

En contraste a la respuesta de IGF-1 en la condición 5.5 mmol/l de glucosa con GH a 10 ng/ml, observamos como en la condición de esteatosis (25mmol/l), independientemente de la dosis de GH administrada no hay incremento en la síntesis de IGF-1. Esta condición pudiera en parte explicarse por el mecanismo con el que se generó la esteatosis en este modelo celular. Si bien sabemos que en un sistema *in vivo* con mecanismos de retroalimentación las condiciones de hiperglucemia primordialmente reducirían la secreción de GH al incrementar el tono somatostatinérgico, pareciera que la sóla condición de hiperglucemia pudiera alterar la secreción de IGF-1 a nivel hepático, tal como lo describío Shishko y cols en pacientes con Diabetes Mellitus tipo 1, quienes al tener un mal control glucémico reportaban bajos niveles de IGF-1, y que al mejorar su condición glucémica normalizaban sus niveles de IGF-1 (86). Por otro lado quizá la propia esteatosis y la gravedad de la misma sea la única condición necesaria que evita la producción de IGF-1,

como lo ha demostrado Rufinatscha y cols. en pacientes con NASH, en los que a mayor grado de inflamación disminuían progresivamente los niveles de IGF-1 independientemente de la secreción de GH (87). Nuestros datos sugieren que la hiperglucemia *per se* pudiera incrementar el almacenamiento de ácidos grasos en los hepatocitos a través de la regulación negativa en la expresión de IGF-1, aún en presencia de GH a distintas concentraciones; sin embargo, estos datos deben considerarse con cautela, ya que en el contexto clínico, una condición de hiperglucemia se acompaña de hiperinsulinismo compensatorio y/o resistencia a la insulina, variables que este modelo *in vitro* no considera.

En estudios previos se ha atribuido al eje GH - IGF-1 un papel importante en la asociación a NAFLD. Al demostrar que nuestro modelo de esteatosis con GH disminuía los cuerpos lipídicos, nos propusimos evaluar si este hallazgo pudiera estar mediado por la acción de IGF-1. Interesantemente no demostramos una mayor expresión de IGF-1, lo que pareciera sugerir que los efectos de GH a nivel hepático son independientes a su principal efector. Estos hallazgos ya habían sido reportados por Zhongbo Liu y cols, en un modelo animal con resistencia a GH en los que se generó NAFLD, que al ser sustituidos hormonalmente con IGF-1 mejoraron su condición de sensibilidad a insulina y disminuyeron la adiposidad visceral, pero no lograron disminuir el estrés oxidativo y la inflamación causadas por esteatosis, lo que indica que la GH y su señalización son factores protectores independientes a IGF-1 para el tejido hepático, tal como puede apreciarse en la enfermedad de Laron que cursa con deficiencia de IGF-1 secundaria a la insensibilidad primaria de GH (88,89).

Se sabe que parte de los mecanismos responsables de la acumulación intrahepática de ácidos grasos es una desbalance entre las vías de lipogénesis y de lipólisis (31). La lipogénesis tiene como mecanismo de activación la expresión de dos principales genes ChREBP y SREBP1c, activados por carbohidratos e insulina respectivamente. ChREBP tiene la capacidad de incrementar la expresión de otros genes involucrados en la lipogénesis, principalmente FAS, lo que se ha documentado en pacientes y en modelos animales con NAFLD (90,91). Acorde con esto, nuestro modelo de esteatosis también presenta una mayor expresión e incremento de la síntesis proteica de ChREBP y FAS,

aunque no SREBP1c, lo cual podría explicarse por el estímulo con el cual se generó la esteatosis (altas concentraciones de glucosa), pero sin cambios en la insulina, que es el factor encargado de incrementar la expresión de SREBP1c. En cuanto al efecto de GH pudimos corroborar que muestra un efecto protector en NAFLD, ya que al administrar dosis crecientes de GH al modelo de esteatosis, tanto ChREBP como FAS disminuyen significativamente su expresión y síntesis, ratificando los hallazgos de microfotografía y cuantificación de triglicéridos. Por otro lado, los genes y proteínas primordiales en la β-oxidación no tuvieron cambios en la condición de esteatosis ni a la administración de GH. El mecanismo de oxidación lipídica que se ha atribuido a GH se ha descrito principalmente en tejido adiposo, y cada vez hay mayor evidencia sobre la divergencia tejido-específica en la respuesta metabólica a GH (92). Nuestros hallazgos sugieren que el papel de GH en NAFLD *in vitro* está mediado primordialmente por la modulación de la lipogénesis *de novo*.

Así, nuestros resultados nos permiten poner en consideración las siguientes conclusiones:
1. Este trabajo sugiere que la alta concentración de glucosa induce esteatosis por la inhibición de la síntesis de IGF-1 a nivel hepático y una mayor actividad de la lipogénesis *de novo*.
2. Pareciera que la modulación de la lipogénesis *de novo* en NAFLD como estrategia terapéutica pudiera mejorar la condición de esteatosis en un modelo *in vitro*.
3. La hormona de crecimiento disminuye la esteatosis (mediada por altas concentraciones de glucosa) en un modelo celular *in vitro* inhibiendo la lipogénesis *de novo* mediante la regulación negativa en la expresión de ChREBP y FAS en una forma dosis-dependiente e independiente a IGF-1. Este hallazgo es interesante, ya que dicho mecanismo pudiera contribuir a modular la esteatosis en pacientes sin deficiencia de GH, como aquellos con obesidad, diabetes o síndrome metabólico, quienes podrían beneficiarse del tratamiento con GH.

Es importante aclarar, que si bien las propuestas en dosis de GH intentan simular las concentraciones encontradas en seres humanos mediante una prueba de estimulación de la misma, no se podría extrapolar como una propuesta de dosificación en pacientes con

NAFLD con o sin deficiencia de GH, y es necesario realizar estudios clínicos para evaluar la factibilidad de su aplicación, y en su momento la respuesta y seguridad para esta indicación en seres humanos.

Estos hallazgos sugieren como mecanismos moleculares de esteatosis la inhibición en la producción de IGF-1 en condiciones de hiperglucemia y la lipogénesis *de novo* como principales mecanismos generadores de NAFLD en pacientes con obesidad, diabetes y síndrome metabólico.

CONCLUSIONES

1. La hiperglucemia induce esteatosis por la inhibición de la síntesis de IGF-1 en hepatocitos y una mayor actividad de la lipogénesis *de novo* en el modelo *in vitro* HepG2.

2. La modulación de la NAFLD por GH pudiera mejorar la condición de esteatosis principalmente por lipogénesis *de novo*.

3. La hormona de crecimiento mejora la esteatosis en el modelo celular *in vitro* HepG2, inhibiendo la lipogénesis *de novo* mediante la supresión de la síntesis de ChREBP y FAS en una forma dosis-dependiente, de forma independiente a IGF-1.

PERSPECTIVAS

Los resultados de este trabajo nos permiten hipotetizar que en seres humanos la asociación entre la disfunción del eje GH-IGF-1 y la esteatosis hepática pudiera estar igualmente mediada por lipogénesis de novo, en un inicio valdría la pena evaluar en un estudio transversal en pacientes con NAFLD la función del eje GH-IGF-1 y valorar si existe una relación inversamente proporcional a la actividad de lipogénesis de novo (estudio dinámico *in vivo*). Es necesario realizar ensayos clínicos controlados en sujetos con NAFLD en los que se pueda plantear un uso metabólico de la GH probablemente mediante la titulación de IGF-1 a fin de conocer su utilidad, eficacia y seguridad.

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ANEXOS

ARTÍCULOS PUBLICADOS DURANTE LOS ESTUDIOS DE POSGRADO

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Growth hormone ameliorates high glucose-induced steatosis on *in vitro* cultured human HepG2 hepatocytes by inhibiting *de novo* lipogenesis *via* ChREBP and FAS suppression

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ABSTRACT

Objective: Growth hormone (GH) deficiency has been associated with increased steatosis but the molecular mechanism has not been fully elucidated. We investigated the effect of GH on lipid accumulation of HepG2 cells cultured on an *in vitro* steatosis model and examined the potential involvement of insulin-like growth factor 1 (IGF-1) as well as lipogenic and lipolytic molecules.

Methods: Control and steatosis conditions were induced by culturing HepG2 cells with 5.5 or 25 mmol/l glucose for 24 h, respectively. Afterward, cells were exposed to 0, 5, 10 or 20 ng/ml GH for another 24 h. Lipid content was quantified as well as mRNA and protein levels of IGF-1, carbohydrate responsive element-binding protein (ChREBP), sterol regulatory element-binding protein 1c (SREBP1c), fatty acid synthase (FAS), carnitine palmitoyltransferase 1A (CPT1A), and peroxisome proliferator-activated receptor alpha (PPAR-alpha) by qPCR and western blot, respectively. Data were analyzed by one-way ANOVA and the Games-Howell post-hoc test.

Results: In the steatosis model, HepG2 hepatocytes showed a significant 2-fold increase in lipid amount as compared to control cells. IGF-1 mRNA and protein levels were significantly increased in control cells exposed to 10 ng/ml GH, whereas high glucose abolished this effect. High glucose also significantly increased both mRNA and protein of ChREBP and FAS without having effect on SREBP1c, CPT1A and PPAR-alpha. However, GH inhibited ChREBP and FAS production, even in HepG2 hepatocytes cultured under steatosis conditions.

Conclusions: Growth hormone ameliorates high glucose-induced steatosis in HepG2 cells by suppressing *de novo* lipogenesis *via* ChREBP and FAS down-regulation.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by hepatic fat accumulation and encompasses a wide spectrum of liver disorders progressively ranging from simple steatosis to steatohepatitis, liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [1]. NAFLD is now considered the most common liver disease worldwide, and due to increasing rates of obesity, NAFLD might be soon the most frequent indication for liver transplantation in west countries [2]. For this reason, it is of great importance to understand the main mechanisms leading to hepatic fat accumulation, with special emphasis on simple steatosis as the first stage of NAFLD development and progression.

Growth hormone (GH), also referred to as somatotropin, is a 191-

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the activity of carnitine palmitoyltransferase 1A (CPT1A) and peroxisome proliferator-activated receptor alpha (PPAR-alpha) [26–28].

Although NAFLD has been thought to associate with increased lipogenesis and decreased fatty acid beta-oxidation [29,30], the exact molecular mechanism orchestrating hepatic fat storage in the presence of GH is not fully understood [31]. We thus wanted to investigate the effect of GH on human HepG2 hepatocytes cultured in the presence of high glucose concentrations as an *in vitro* steatosis model, also examining the possible involvement of lipogenic and lipolytic molecules.

Material and methods

HepG2 cell propagation and culture.

HepG2 cell line was obtained from the American Tissue Culture Collection (ATCC®, Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher-Scientific, USA) containing 10% fetal bovine serum (FBS), 10 nM HEPES buffer (Sigma-Aldrich, St. Louis, MO), and 50 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO) at 37 °C in humidified 5% CO_2 atmosphere. Once HepG2 cells reached about 80% confluence, they were rinsed twice using PBS1X and detached in the presence of 0.05% trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO) for 5 min. After 10 min of centrifugation at 200g/8 °C, HepG2 cells were gently resuspended in DMEM supplemented as mentioned before and placed in 6-well cell-culture plates (Costar, USA) at a density of 5×10^4 cells per well for 24 h to allow cell adherence. Afterwards, HepG2 cells were used for *in vitro* culture experiments. For passage HepG2 cells, culture media were replaced every other day before getting 70% confluence. For *in vitro* culture experiments, HepG2 cells were used every five passages.

In vitro steatosis model.

HepG2 cells were seeded in DMEM containing either 5.5 (control cells) or 25 mmol/l D-glucose (steatosis model), and 10% FBS, 10 nM HEPES buffer and 50 μg/ml gentamicin at 37 °C in humidified 5% CO_2 atmosphere using 6-well cell-culture plates at a density of 5×10^4 cells per well for 24 h. Then, HepG2 cells were exposed to 0, 5, 10, or 20 ng/ml human recombinant growth hormone (Peprotech, Mexico) for another 24 h (Fig. 1). *In vitro* culture experiments were performed in five independent replicates.

Histological quantification of lipid droplets.

After 24 h, HepG2 cells cultured in the presence of 0, 5, 10, or 20 ng/ml human recombinant growth hormone were rinsed twice using PBS1X and fixed with 4% p-formaldehyde for 30 min at room temperature. After 15 min permeabilization using 0.01% saponin and PBS1X supplemented with 0.1% BSA, attached HepG2 cells were stained with Oil Red O solution (0.5 g Oil Red O powder dissolved in 70% ethanol, Sigma-Aldrich, St. Louis, MO) for 20 min at room temperature. Then, attached HepG2 cells were rinsed with PBS1X and lipid droplets counted per 100 cells in 10 high-powered fields from each culture well. Microphotographs were acquired at 40× magnification using a Nikon Microphot-FXA microscope coupled to a Nikon Digital Camera DXM1200F.

Quantification of the intracellular triglyceride content.

HepG2 cells cultured in the presence of 0, 5, 10, or 20 ng/ml human recombinant growth hormone were rinsed twice using PBS1X and digested using 250 μl lipase for 4 min (Sigma-Aldrich, St. Louis, MO). After stopping the enzymatic digestion, protein concentration was quantified by absorbance at 595 nm using the Bradford Protein Assay (Bio-Rad, USA). At the same time, triglyceride concentration was measured by incubating the cell suspension in the presence of 0.4 ml methanol/0.5 ml chloroform for 45 min. Then, 0.8 ml of 100 mM sodium chloride was added and the fraction containing triglycerides was dried at 45 °C for 30 min. Afterwards, 50 μl triglyceride standard solution (Abcam, Cambridge, UK) was added and triglyceride concentration quantified by spectrometry at 570 nm using a microplate reader.

Total RNA isolation.

HepG2 cells cultured in the presence of 0, 5, 10, or 20 ng/ml human recombinant growth hormone for 24 h were detached with 0.05% trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO) for 5 min. After 15 min of centrifugation at 200g/8 °C, HepG2 cells were disrupted in 0.5 ml TRIzol reagent (Invitrogen, Carlsbad, CA) at 4 °C for 1 min. Then, 0.2 ml cold chloroform (Sigma-Aldrich, St. Louis, MO) per 1 ml cell suspension was added to each sample and incubated at 4 °C/15 min. After 15 min of centrifugation at 20800g/4 °C, the aqueous phase was recovered and placed into 1.6 ml Eppendorf tubes (Axygen, Mexico). Afterwards, total RNA was precipitated with 0.8 ml isopropyl alcohol (J.T. Baker, Mexico) at −20 °C for 2 h. Immediately after, RNA samples were centrifuged at 20800g/4 °C for 15 min and supernatant discarded. Subsequently, RNA pellets were rinsed thrice with 100% absolute ethanol (J.T. Baker, Mexico) and resuspended in RNAse-free water treated with DEPC (Sigma-Aldrich, St. Louis, MO). Total RNA concentration was measured by absorbance at 260/280 nm and RNA integrity verified by electrophoresis on 1.0% denaturing agarose gel (Promega, Uniparts, Mexico) in the presence of 2.2 mmol/l p-formaldehyde.

Evaluation of the mRNA levels of ChREBP, SREBP1c, FAS, CPT1A, PPAR-alpha, and IGF-1 by qPCR.

Total RNA samples from HepG2 cells were reverse-transcribed by using the M-MLV retrotranscriptase system and dT primer (Invitrogen, Carlsbad, CA). cDNA was specifically amplified by quantitative polymerase chain reaction (qPCR) using human-specific primers to detect mRNA levels of ChREBP, SREBP1c, FAS, CPT1A, PPAR-alpha, and IGF-1 (Table 1). Briefly, cDNA samples (100 ng/μl) were individually placed in the BIO-RAD CFX96 Real-Time System thermal cycler in the presence of PCR supermix that consisted in 7.5 ml SsoAdvanced Universal SYBR Green Supermix (BIO-RAD, USA), 0.6 μl specific-primer solution, 5.3 μl water, and 1 μl DNA sample per tube. The 18S-ribosomal RNA primer was used as an endogenous gene for ΔΔCT calculation and normalization (Table 1) and results expressed as fold change.

Measurement of IGF-1 by ELISA.

Table 1
Primer sequences for qPCR amplification.

Gene	Forward Primer	Reverse Primer	Product Length (bp)
IGF-1	5′- TGGTGGACGCTCTTCAGTTC -3′	5′- TCCGGAAGCAACACTCATCC -3′	123
ChREBP	5′- CCGTCCAGACAGCAACAAGA −3′	5′- GCTGCTGGCACAGGTTAATG −3′	275
FAS	5′- TGCGTGGCCTTTGAAATGTG −3′	5′- CTCCATGTCCGTGAACTGCT −3′	260
SREBP1c	5′- GATGCGGAGAAGCTGCCTAT −3′	5′- GCTGTGTTGCAGAAAGCGAA −3′	234
CPT1A	5′- GACGGCTATGGTGTGTCGTA −3′	5′- TCCAGCCCAGCACATGAAC -3′	202
PPAR a	5′- ACACCGAGGACTCTTGCGA −3′	5′- GGAAAGGGCAAGTCCCGATG −3′	207
GAPDH	5′- AAAGCCTGCCGGTGACTAAC -3′	5′- TTCCCGTTCTCAGCCTTGAC -3′	300

Abbreviations: IGF-1, insulin-like growth factor 1; ChREBP, carbohydrate responsive element-binding protein; SREBP1c, sterol regulatory element-binding protein 1c; FAS, fatty acid synthase; CPT1A, carnitine palmitoyltransferase 1A; PPAR-alpha, peroxisome proliferator-activated receptor alpha; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; bp, base pairs.

Culture supernatants were collected into pyrogen-free 2.0 ml Eppendorf tubes (Axygen, Mexico) and stored at −80 °C until use. IGF-1 supernatant levels were quantified by the Enzyme-Linked ImmunoSorbent Assay (ELISA), following the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO).

Assessment of the protein levels of ChREBP, SREBP1c, FAS, CPT1A, and PPAR-alpha by western blot.

HepG2 cells were disrupted in protein extraction buffer that contained 500 mmol/l Tris-HCl (1 ml/50 × 10^4 cells) and protease inhibitor cocktail (Calbiochem, Germany). After a centrifugation step at 20800g/8 °C for 15 min, supernatants were recovered from cell suspensions and protein concentration quantified by absorbance at 595 nm by means of the Bradford Protein Assay (Bio-Rad, USA). Immediately after, 30 μg of total protein was boiled in Laemmli sample buffer and separated using SDS-PAGE with 10% acrylamide. Then, proteins were transferred to PVDF membranes (Thermo Fisher Scientific, Inc., USA) that in turn were blocked with PBS 1× buffer (0.2% Tween 20) containing 1% BSA for 12 h. After being rinsed five times with PBS 1×-Tween 20, PVDF membranes were incubated in the presence of 1:500 rabbit anti-human ChREBP (95 KDa), 1:100 rabbit anti-human SREBP1c (132 KDa), 1:1000 rabbit anti-human FAS (273 KDa), 1:250 rabbit anti-human CPT1A (88 KDa), or 1:150 rabbit anti-human PPAR-alpha (52 KDa) (Abcam, Cambridge, UK) for 60 min at room temperature. The peroxidase-diaminobenzidine reaction (Sigma-Aldrich, Mexico) was used to visualize the protein bands that in turn were quantified by optical density (OD) using β-actin as control protein.

Statistics

Data were obtained from five independent replicates. Normality of data was evaluated using the Shapiro-Wilk test. Data were analyzed using one-way ANOVA followed by the post-hoc Games-Howell test to estimate significant differences among HepG2 cells treated with different concentrations of human recombinant growth hormone for 24 h in terms of mRNA and protein levels of ChREBP, SREBP1c, FAS, CPT1A, PPAR-alpha, and IGF-1 as well as lipid droplet amount. Data are expressed as mean ± standard deviation and differences considered significant when $P < 0.05$. Results were analyzed and graphed in the GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA 92037 USA).

Results

Flow chart of the experimental design to generate the *in vitro* steatosis model can be seen in Fig. 1.

In normal glucose concentrations (5.5 mmol/l), control HepG2 hepatocytes without exposure to GH showed some positive signal for oil red staining (Fig. 2A). Notably, HepG2 cells treated with 5 ng/ml GH exhibited less number of lipid droplets than that found in untreated HepG2 hepatocytes (Fig. 2B). However, the positive signal for oil red staining tended to remain unaltered in HepG2 cells treated with 10 and 20 ng/ml GH as compared to untreated hepatocytes (Fig. 2C and D, respectively). On the contrary, HepG2 cells cultured in the presence of 25 mmol/l glucose dramatically increased the positive signal for oil red staining with regard to HepG2 hepatocytes cultured in normal glucose concentrations (Fig. 2E). Interestingly, exposure of HepG2 hepatocytes to 5 ng/ml GH tended to reduce the amount of intracellular lipid droplets even in the *in vitro* steatosis model (25 mmol/l glucose) (Fig. 2F). In the same sense, the effect of GH on reducing the lipid content in HepG2 cells cultured with 25 mmol/l glucose was even more pronounced when using 10 or 20 ng/ml GH than 5 ng/ml (Fig. 2G and H, respectively).

The apparent reduction in fat storage of HepG2 hepatocytes cultured in the presence of GH was confirmed by quantifying the number of lipid droplets per cell and the intracellular triglyceride content. Untreated HepG2 hepatocytes cultured in normal glucose concentrations showed 14.81 ± 12.35 lipid droplets per cell (Fig. 3A, left side panel). In the same *in vitro* culture conditions, HepG2 cells exposed to 5, 10 or 20 ng/ml GH exhibited 8.33 ± 14.64, 17.21 ± 14.41, and 11.56 ± 10.88 lipid droplets per cell, respectively (Fig. 3A, left side panel). In contrast, HepG2 hepatocytes cultured in the presence of 25 mmol/l glucose showed a significant 2-fold increase in the lipid content per cell (29.07 ± 23.09) as compared to untreated HepG2 cells (Fig. 3A, right side panel). Notably, HepG2 hepatocytes cultured in steatosis conditions exhibited significant 2.3, 7.6, and 4-fold reductions in the number of lipid droplets per cell when treated with 5, 10 or 20 ng/ml GH, respectively, as compared to cells also cultured with 25 mmol/l glucose without GH exposure (Fig. 3A, right side panel). Quantification of the intracellular triglyceride content confirmed the effect of GH on fat storage in HepG2 cells. As a matter of fact, no significant increases in lipid content was found in HepG2 hepatocytes cultured in the presence of GH, irrespectively of having been exposed to different hormone concentrations (Fig. 3B, left side panel). On the contrary, exposure of HepG2 cells to high glucose induced a significant 25% increase in the intracellular triglyceride content as compared to that found in cells cultured in normal glucose conditions (Fig. 3B, right side panel). Confirming the apparently protective role of GH in hepatic fat storage, HepG2 hepatocytes cultured in steatosis conditions showed progressive diminutions in the amount of intracellular triglycerides when treated with 5, 10 or 20 ng/ml GH (Fig. 3B, right side panel).

The possible molecular mechanism by which GH ameliorates steatosis on *in vitro* cultured HepG2 cells was also explored. IGF-1 is the main mediator of the anabolic and mitogenic effects of GH in mammals. In normal glucose conditions (5.5 mmol/l glucose), exposure of HepG2 cells to 10 ng/ml GH induced a significant 1.7-fold increase in IGF-1 expression as compared to that found when used 5 ng/ml GH (Fig. 4A, left side panel). Notably, a negative feedback was seen in the regulation of IGF-1 expression when HepG2 hepatocytes were treated with 20 ng/ml GH (Fig. 4A, left side panel). On the contrary, high glucose conditions of the *in vitro* steatosis model (25 mmol/l glucose) tended to suppress IGF-1 expression in HepG2 cells, independently of having been exposed to different GH concentrations (Fig. 4A, right side panel). As a matter of fact, high glucose levels abolished even the stimulatory effect of 10 ng/ml GH upon IGF-1 expression in HepG2 hepatocytes cultured on normal glucose concentrations (Fig. 4A, right side panel). IGF-1 protein levels showed similar results than those found when studied mRNA (Fig. 4B). In fact, HepG2 hepatocytes cultured with normal glucose showed a significant 17-fold increase in IGF-1 protein secretion when treated with 10 ng/ml GH as compared to untreated control cells (Fig. 4B, left side panel). Once again, HepG2 cells treated with 20 ng/ml GH exhibited no significant changes in IGF-1 production, which confirmed that 20 ng/ml GH has a negative regulatory effect on IGF-1 (Fig. 4B, left side panel). On the other hand, HepG2 hepatocytes cultured under steatosis conditions (25 mmol/l glucose) showed no significant changes in IGF-1 protein levels, independently of having been exposed to different GH concentrations (Fig. 4B, right side panel).

ChREBP expression is activated by glucose in skeletal muscle and liver. In normal glucose levels, HepG2 hepatocytes did not show ChREBP expression, irrespectively of having been exposed to different GH concentrations (Fig. 5A, left side panel). As expected, HepG2 cells exposed to 25 mmol/l glucose showed a significant 3.5-fold increase in ChREBP expression in the absence of GH (Fig. 5A, right side panel). Notably, the use of 5 ng/ml GH diminished ChREBP expression to normal values in HepG2 hepatocytes cultured in the *in vitro* steatosis model (Fig. 5A, right side panel). In the same *in vitro* culture conditions, exposure of HepG2 cells to 10 ng/ml GH induced 50% reduction in ChREBP expression as compared to that found when used 5 ng/ml GH (Fig. 5A, right side panel). HepG2 cells treated with 20 ng/ml GH showed a trend to reduce ChREBP expression as compared to that found when used 5 ng/ml GH, even though no significant differences were seen (Fig. 5A, right side panel).

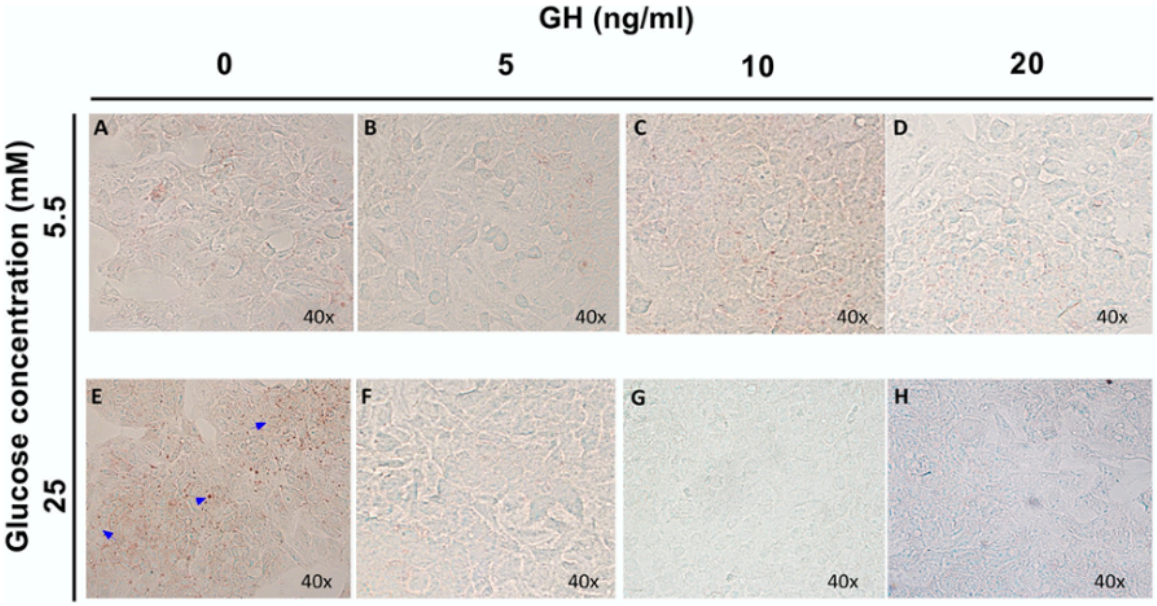

Fig. 2. Microphotographs showing HepG2 cells cultured with 5.5 or 25 mmol/l glucose in the presence or absence of growth hormone. (A) In control culture conditions (5.5 mmol/l glucose), HepG2 hepatocytes cultured in the absence of growth hormone (GH) showed scarce number of lipid droplets. (B) HepG2 cells treated with 5 ng/ml GH exhibited less number of lipid droplets than that found in untreated HepG2 hepatocytes. The lipid droplet content diminished even more in HepG2 cells treated with 10 or 20 ng/ml GH than untreated HepG2 hepatocytes (C and D, respectively). (E) In the steatosis model (25 mmol/l glucose), HepG2 cells cultured in the absence of GH dramatically increased the number of lipid droplets with regard to HepG2 cells cultured with 5.5 mmol/l glucose. (F) Exposure of HepG2 hepatocytes to 5 ng/ml GH tended to reduce the amount of lipid droplets, even in the *in vitro* steatosis model. The effect of GH on reducing the lipid content in HepG2 cells cultured with 25 mmol/l glucose was even more pronounced when using 10 or 20 ng/ml GH than 5 ng/ml (G and H, respectively). In order to have a semiquantitative measure of the amount of intracellular fat, lipid droplets were visualized using Oil-Red staining and counted per cell in 100 cells contained in 10 high-powered fields from each culture well. Microphotographs were acquired at 40× magnification using a Nikon Microphot-FXA microscope coupled to a Nikon Digital Camera DXM1200F.

SREBP1c is a lipogenic gene that is expressed in response to insulin in liver tissue. In normal glucose concentrations, HepG2 hepatocytes did not show significant differences in SREBP1c expression, regardless the simultaneous exposure of these cells to different GH concentrations (Fig. 5B, left side panel). Similarly, HepG2 cells cultured in the *in vitro* steatosis model did not show significant changes in SREBP1c expression, irrespectively of having been exposed to different GH concentrations (Fig. 5B, right side panel).

FAS expression is the decisive step in lipid production in adipose tissue and liver. In normal glucose levels, HepG2 hepatocytes showed low FAS expression, independently of having been treated with 10 or 20 ng/ml GH (Fig. 5C, left side panel). However, the use of 5 ng/ml GH induced a mild but significant 3.5-fold increase in FAS expression in HepG2 cells cultured in normal glucose concentrations (Fig. 5C, left side panel). As expected, HepG2 hepatocytes exposed to 25 mmol/l glucose showed an extraordinary 17-fold increase in FAS expression in the absence of GH (Fig. 5C, right side panel). It is worth mentioning that the use of 5, 10 or 20 ng/ml GH totally abolished FAS expression in HepG2 cells cultured in the *in vitro* steatosis model with regard to that found in the absence of GH (Fig. 5C, right side panel).

CPT1A expression is a key factor in fatty acid beta-oxidation. In normal glucose concentrations, HepG2 hepatocytes tended to increase CPT1A expression when exposed to 5 ng/ml GH as compared to HepG2 cells cultured in the absence of GH. However, no significant changes were found (Fig. 6A, left side panel). Likewise, no significant changes in CPT1A expression were found in HepG2 hepatocytes exposed to 10 or 20 ng/ml GH with respect to HepG2 cells that were not treated with GH (Fig. 6A, left side panel). In the same sense, no significant changes were found in CPT1A expression when HepG2 hepatocytes were cultured in high glucose levels, irrespectively of having been exposed to different GH concentrations (Fig. 6A, right side panel).

PPAR-alpha expression is also a pivotal mediator of lipolysis. However, neither high glucose conditions nor GH concentrations had significant effects on PPAR-alpha expression in HepG2 hepatocytes (Fig. 6B).

Fig. 7A illustrates a representative poly-acrylamide gel showing specific protein bands for ChREBP, SREBP1c, FAS, CPT1A, and PPAR-alpha from HepG2 hepatocytes cultured under normal or steatosis conditions, in the presence of different GH concentrations. In normal glucose conditions, GH did not induce ChREBP protein synthesis at any concentration (Fig. 7B, left side panel). On the contrary, ChREBP protein levels exhibited a significant 2-fold increase in HepG2 hepatocytes cultured with high glucose in the absence of GH (Fig. 7B, right side panel). The use of 5 and 10 ng/ml GH significantly reduced the stimulatory effect of high glucose on ChREBP protein production and reached a maximum response when HepG2 hepatocytes were treated with 20 ng/ml GH (Fig. 7B, right side panel). Concurring with mRNA expression levels, SREBP1c protein production did not show significant changes in HepG2 hepatocytes cultured in control or steatosis conditions, independently of having been exposed to different GH concentrations (Fig. 7C). In normal glucose conditions, GH did not induce significant changes in the amount of FAS protein at any concentration (Fig. 7D, left side panel). However, exposure of HepG2 cells to high glucose promoted a significant 4-fold increase in FAS protein production when cultured in the absence of GH (Fig. 7D, right side panel). Notably, the stimulatory effect of high glucose on FAS synthesis was clearly abolished when HepG2 hepatocytes were treated with 5, 10 or 20 ng/ml GH (Fig. 7D, right side panel). Confirming the mRNA expression results, HepG2 cells cultured with normal or high glucose did not show significant changes in the protein levels of CPT1A or PPAR-

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Association between neck circumference and non-alcoholic fatty liver disease in Mexican children and adolescents with obesity

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Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic hepatic disorder in the pediatric population and has grown along with the obesity pandemic in which we live today. Adipose tissue storage in the upper body segment has been positively correlated with visceral adiposity and metabolic disease, which suggests that neck circumference could represent an easily accessible and replicable anthropometric measurement to identify patients with a higher risk of developing NAFLD. The main purpose of this study is to determine if there is an association between neck circumference and NAFLD. The secondary objectives are to establish cutoff values based on gender and puberty staging.

Methods: We included a sample pediatric population of 112 patients diagnosed with obesity aged between 6 and 18 years. We performed anthropometric and metabolic measurements on every patient, and NAFLD diagnosis was determined with hepatic ultrasound.

Results: The neck circumference was larger in NAFLD pediatric patients compared to those without NAFLD ($p=0.001$). In a multivariate analysis, the neck circumference was associated with NAFLD as an independent risk factor (odds ratio [OR] = 1.172; 95% CI = 1.008–1.362; $p=0.038$). Tanner 2–3 = 35 cm and Tanner 4–5 = 38 cm were established as risk cutoff values to develop NAFLD in the male adolescent population.

Conclusions: There is an association between the neck circumference and NAFLD in pediatric patients with obesity, particularly in the male population.

Keywords: childhood obesity; neck circumference; non-alcoholic fatty liver disease; obesity.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a term used to describe a wide disease spectrum that ranges from simple steatosis to non-alcoholic steatohepatitis (NASH). NAFLD is characterized by presenting steatosis of the hepatic parenchyma greater than 5% without previous history of alcohol consumption or other known liver diseases [1, 2]. The increasing prevalence of this disease has been associated with the obesity pandemic that has appeared in the last three decades and the addition of high fructose consumption into our diets [3].

NAFLD is the most common chronic hepatic disorder in children, with a prevalence of 10% in the general

pediatric population and 40–70% in pediatric populations with obesity [3, 4]. The risk factors that are associated with NAFLD in this age group are male gender, pubertal stage and Hispanic ethnicity [2, 5].

The greatest risk factor for NAFLD and other metabolic diseases, particularly in the male gender, could be explained by the abdominal and trunk adipose tissue distribution. It is widely known that abdominal adiposity, particularly visceral adipose tissue (VAT), is an independent risk factor for developing cardiac and metabolic diseases [6, 7].

The characteristics and distribution of subcutaneous adipose tissue (SAT) have been less studied than VAT. However, it is known that while the gluteo-femoral region SAT has energy storage properties that do not increase metabolic risk, the upper body region SAT, including neck adipose tissue (NAT), seems to be similar to VAT, increasing the risk of developing metabolic diseases [6].

Neck circumference (NC) has been suggested as an easily accessible and replicable anthropometric measurement to perform NAT evaluation [8], which has previously shown associations to body mass index (BMI) and VAT [9–12] and is also an independent risk factor for central obesity and metabolic disease [13–16].

There are limited studies regarding the association between NC and NAFLD, and most of them deal only with adult populations. However, the few studies performed in child population with obesity have shown positive correlations, with differences between male and female genders [17–19].

The purpose of this study was to determine if there is an association between NC and NAFLD in Mexican children with obesity and to establish cutoff values based on gender and puberty staging.

Materials and methods

This is a cross-sectional comparative study that was approved by the Research Ethics Committee, Hospital General de Mexico "Dr. Eduardo Liceaga". We got informed consent and assent from all individuals and their parents/legal guardians in this study. All personal health information used has been in compliance with all the relevant national regulations, institutional policies and under the tenets of the Helsinki Declaration. This research has been approved by the authors' institutional review board (DIR/17/311/03/010).

We analyzed a sample of a pediatric population including 112 patients (49 girls and 63 boys, aged between 6 and 18 years) diagnosed with obesity (determined with the percentile BMI ≥ 95 according to the criteria established by the Centers for Disease Control and Prevention [CDC]) from the Children and Adolescent Obesity Clinic, Hospital General de Mexico "Dr. Eduardo Liceaga", Mexico City, Mexico. We excluded patients who had obesity of genetic or endocrine origin, previous medical history of viral or congenital hepatitis, history of alcohol consumption or chronic use of medically approved drugs or alternative medicine. None of our patients had a previous history of type 2 diabetes when NAFLD was diagnosed. We performed and documented a complete medical record for each patient included in this study.

Metabolic and anthropometric evaluation

Pediatricians or pediatric endocrinologists performed all of the medical evaluations and anthropometric measurements.

We measured the height and weight with a standardized mechanical weighing scale and a stadiometer using the least amount of clothing as possible. We measured the blood pressure using a manual sphygmomanometer with a cuff size according to age on the right arm of the patient. The NC was measured by a tape applied around the inferior margin of the laryngeal prominence and perpendicular to the long axis of the neck, and the minimum circumference was recorded to the nearest 0.1 cm [10]. Tanner staging was determined during the physical evaluation of pubertal development [20, 21].

Blood samples were collected after a 12-h fasting period and were analyzed at the clinical laboratory of the "Hospital General de Mexico" to determine glucose, insulin, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT).

BMI was calculated using the Quetelet formula [22], and its respective percentile was determined by the Epi Info 3.5.4 Software (CDC, Atlanta, GA, USA). We calculated the insulin resistance index using the *homeostasis model assessment* (HOMA-IR) formula [23].

Ultrasound

An acoustic radiation force impulse (ARFI) hepatic elastography and mode B Doppler abdominal ultrasound was done in every patient. We performed these imaging studies with high-energy and low-duration pulses with a 1–4 MHz convex transducer on a SIEMENS ACUSON S2000 ultrasound system (Siemens, Mountain View, CA, USA). We made ultrasonographic exploration in the supine and left lateral decubitus position. The elastography was performed placing the transducer between the right intercostal spaces on a left lateral decubitus position with the right arm at maximum abduction to enhance the parenchyma visualization. The ultrasound operator fixed the region of interest in a predetermined rectangular dimension (10 mm long, 6 mm wide and with a volume of 2 mm^3) 2 cm below the lower liver margin (Gleason capsule) and a maximum depth of 8 cm from the cutaneous surface, obtaining 10 quantitative samples during the expiration phase and requesting the patients to hold their breath for 4–6 s. The same medical radiologist, who had 28 years of ultrasound experience and 8 years of acoustic radiation force impulse (ARFI) hepatic elastography experience, assessed all of the patients. The propagation speed of the transversal wave, measured in meters per second, was considered as an elasticity marker in the region of interest (Figure 1).

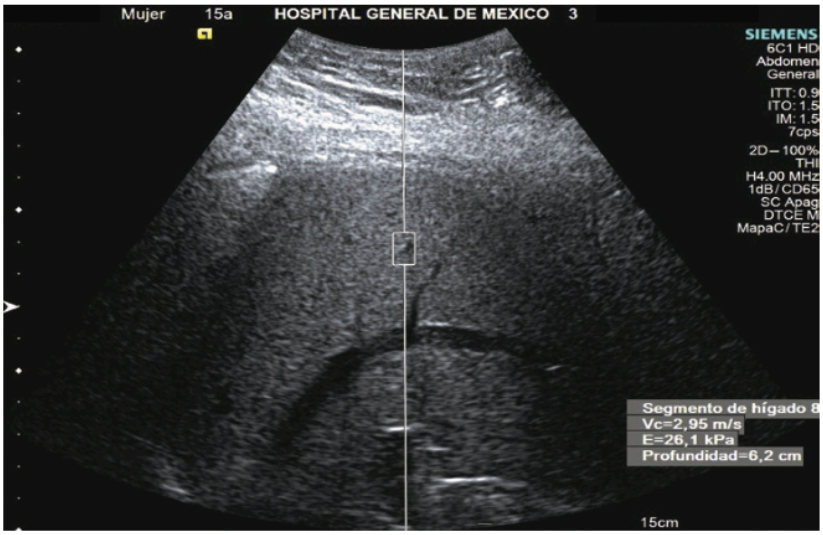

Figure 1: Ultrasound elastography example.

Statistical analysis

We reported the inferential and descriptive statistics for the numerical values as means with standards of deviations and the categorical variables as percentages. We used Student's t test to make inferential statistics to differentiate the group with and without NAFLD according to their gender.

We tested NC for NAFLD through discrimination analysis by receiver operating characteristics (ROC). This test was performed by gender and Tanner stage to obtain the cutoff points for NC. We found that there were similarities between patients with Tanner stages 2 and 3 and those with Tanner stages 4 and 5. Therefore, we grouped these patients to optimize the statistical analysis of our sample population. The chi-squared (χ^2) test evaluated the categorical variables.

We performed the multivariate test through a binomial logistic regression analysis by using NAFLD as the dependent variable and ALT, BMI and NC as independent variables.

We performed all statistical evaluations with the statistical software SPSS version 22 (IBM, Armonk, NY, USA). We considered statistical significance when an alpha error value was <0.05.

Results

Descriptive statistics

In this sample population, we found a NAFLD prevalence of 58% with a 1.24:1 male-to-female ratio. The demographic, biochemical and anthropometric characteristics of the sample population are shown in Table 1.

Inferential statistics

Through mean value comparisons, we found that there were differences between those patients without and with NAFLD (NC = 34.63 ± 3.2 cm vs. 34.85 ± 3.2 cm [p = 0.002]; waist circumference [WC] = 86.71 ± 10 cm vs. 94.61 ± 11.8 cm [p < 0.001]; systolic blood pressure [SBP] = 106.19 ± 14 mmHg vs. 111.36 ± 13.5 [p = 0.05]; LDL-C = 95.44 ± 27.2 mg/dL vs. 105.74 ± 29.5 mg/dL [p = 0.09]; triglycerides = 145.02 ± 86.4 mg/dL vs. 194.58 ± 108.4 mg/dL [p = 0.02]; ALT = 24.21 ± 10.1 IU/L vs. 47.28 ± 31.3 IU/L [p < 0.001]; AST = 26.08 ± 8.9 IU/L vs. 35.83 ± 16.2 IU/L [p = 0.001]; AST/ALT index = 1.14 ± 0.3 vs. 0.88 ± 0.3; HOMA-IR = 3.42 ± 2.9 vs. 4.84 ± 2.7 [p = 0.05] and mean elastography [ARFI] = 1.09 ± 0.33 m/s vs. 1.88 ± 0.7 m/s [p < 0.001], respectively [Table 1]).

We compared mean values by gender between the NAFLD and non-NAFLD groups with Student's t test and found that the anthropometric parameters in the male group had significant differences for NC (p = 0.006); WC (p = 0.003); triglycerides (p = 0.04); ALT (p < 0.001); AST (p = 0.006); AST/ALT ratio (p = 0.001); and uric acid (p = 0.03). The female group only had significant differences in WC (p = 0.04); ALT (p = 0.003); and AST (p = 0.006), GGT (p = 0.02) and HOMA-IR (p = 0.01) (Table 1).

We created ROC curves of NC for NAFLD adjusted by Tanner stage and gender, and they showed a significant statistical difference only between the male adolescents.

Table 1: Anthropometric, metabolic and elastography variables associated with NAFLD in Mexican children with obesity.

	Total			Male			Female		
	NO NAFLD n=47	NAFLD n=65	p-Value	NO NAFLD n=27	NAFLD n=36	p-Value	NO NAFLD n=20	NAFLD n=29	p-Value
Age, years	10.66±3.2	11.97±3.1	0.03	10.41±3.2	11.67±2.9	0.15	11.00±3.4	12.34±3.5	0.18
BMI *z-score*	2.10±0.5	2.18±0.3	0.28	1.90±0.56	2.06±0.6	0.18	2.16±0.2	2.14±0.3	0.82
NC, cm	34.63±3.2	36.77±3.6	0.002	34.85±3.2	37.38±3.7	0.006	34.34±3.4	36.01±3.4	0.10
WC, cm	86.71±10	94.61±11.8	<0.001	87.01±10.1	96.18±12.6	0.003	86.31±10.3	92.67±10.5	0.04
SBP, mmHg	106.19±14.0	111.36±13.5	0.05	107.33±12.7	112.42±14	0.14	104.65±15.7	110.00±13.0	0.20
DBP, mmHg	70.02±9.4	72.91±9.0	0.11	70.67±8.4	73.75±9.5	0.19	69.15±10.8	71.82±8.4	0.34
Total-C, mg/dL	165.4±34.2	169.86±32.4	0.51	168.32±29.6	169.69±28.9	0.86	160.88±40.9	170.08±37.1	0.46
HDL-C, mg/dL	38.49±10.1	36.6±8.2	0.33	36.00±8.8	36.72±8.9	0.77	42.47±11.0	36.43±7.4	0.06
LDL-C, mg/dL	95.44±27.2	105.74±29.5	0.09	100.75±29.3	106.75±30.3	0.47	86.93±21.9	104.29±29.0	0.06
Triglycerides, mg/dL	145.02±86.4	194.58±108.4	0.02	145.12±76.3	203.16±118.5	0.04	144.88±102.9	183.50±95.3	0.23
ALT, U/L	24.21±10.1	47.28±31.3	<0.001	26.63±10.0	53.39±34.7	<0.001	20.33±9.2	38.95±24.1	0.003
AST, U/L	26.08±8.9	35.83±16.2	0.001	28.35±9.8	39.35±17.7	0.006	22.08±5.4	31.05±12.5	0.006
AST/ALT ratio	1.14±0.3	0.88±0.3	<0.001	1.12±0.3	0.85±0.2	0.001	1.16±0.4	0.93±0.3	0.08
GGT, mg/dL	16.25±4.8	24.5±11.3	0.25	16.00±3.7	65.5±87.7	0.34	16.5±6.4	47.00 ±12.4	0.02
Uric acid, mg/dL	5.41±1.3	6.83±5.4	0.14	5.51±1.4	6.55±1.7	0.03	5.25±1.0	7.22±6.2	0.40
Glucose, mg/dL	87.22±8.2	90.39±8.1	0.06	87.2±7.9	90.14±7.6	0.16	87.25±8.9	90.72±8.9	0.23
Insulin, U/L	15.41±11.3	21.37±11.3	0.04	16.54±13.4	19.67±9.8	0.44	13.39±6.1	23.17±12.7	0.02
HOMA-IR	3.42±2.9	4.84±2.7	0.05	3.72±3.5	4.47±2.4	0.47	2.87±1.3	5.24±2.9	0.01
ARFI, m/s	1.09±0.33	1.88±0.7	<0.001	1.12±0.2	1.83±0.9	0.06	1.03±0.1	1.93±0.6	0.09

All values are expressed as mean±standard of deviation. NAFLD, non-alcoholic fatty liver disease; BMI, body mass index *z score*; NC, neck circumference; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase; HOMA-IR, homeostasis model assessment insulin resistance index; ARFI, acoustic radiation force impulse.

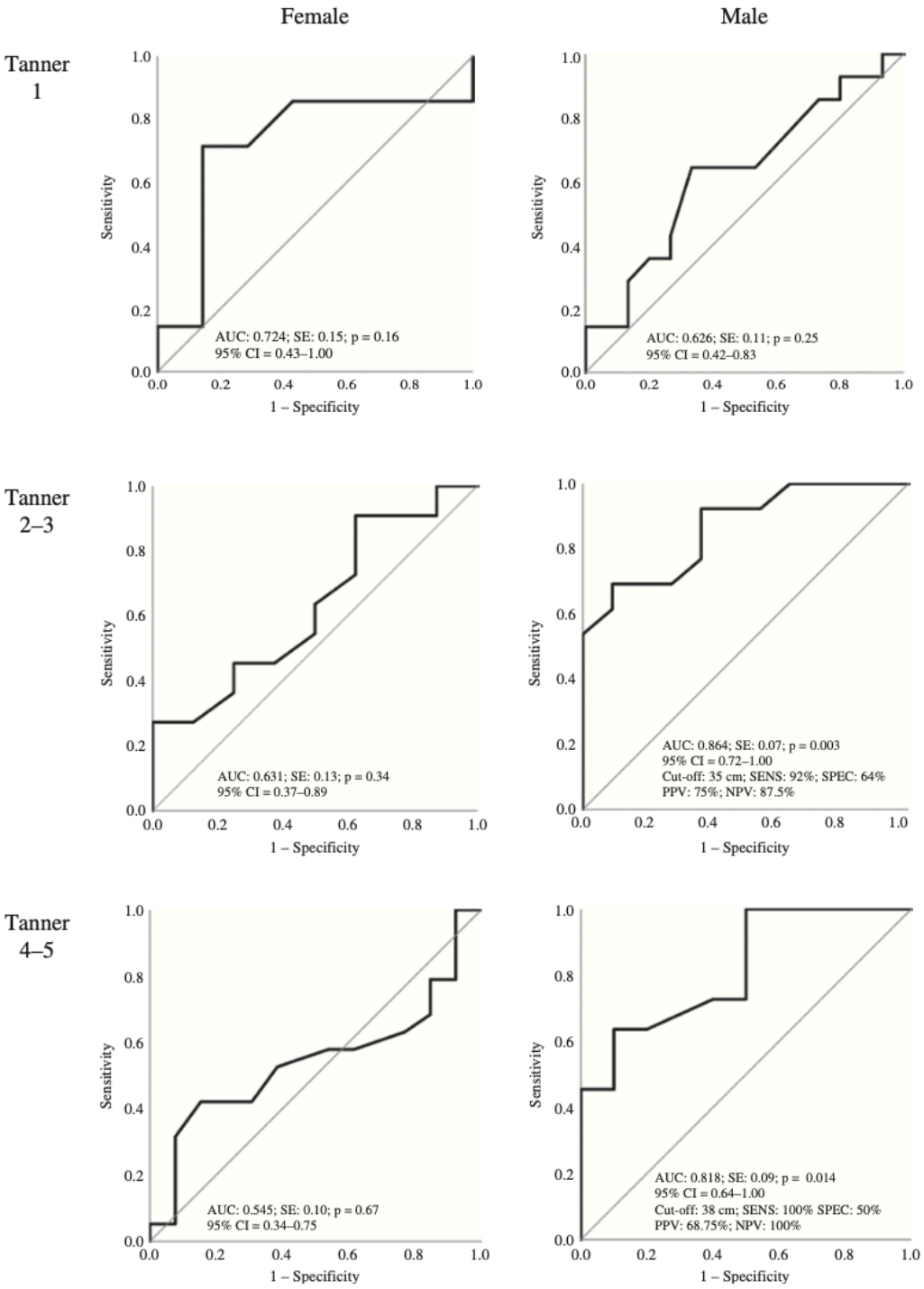

Figure 2: Neck circumference ROC curve by gender and Tanner stage.
AUC, area under the curve; SE, standard error; $p < 0.005$ significance; CI, confidence interval; SENS, sensitiviy; SPEC, specificity; PPV, positive predictive value; NPV, negative predictive value.

Table 2: Logistic regression predicting the likelihood of NAFLD in Mexican children with obesity, based on gender, ALT, NC and BMI *z score*.

	β	Exp (β)	CI (95%)	p-Value
Gender	0.75	2.13	0.75–6.04	0.158
ALT, UI/L	0.07	1.07	1.03–1.12	0.001
NC, cm	0.18	1.19	1.02–1.40	0.031
BMI *z score*	−0.34	0.71	0.15–3.45	0.672

NAFLD, non-alcoholic fatty liver disease; ALT, alanine aminotransferase; NC, neck circumference; BMI *z score*, body mass index *z score*; CI, confidence interval.

This group of males classified as Tanner stages 2–3 were found to be in the area under the curve (AUC) = 0.86 (p = 0.003), and those with stages 4–5 showed an AUC = 0.81 (p = 0.014) (Figure 2). We could not find statistically significant differences in adolescent females.

In Tanner stage 2–3 males, we determined an NC cutoff point of 35 cm (sensitivity = 92%; specificity = 64%; positive predictive value [PPV] = 75%; and negative predictive value [NPV] = 87.5%). In Tanner stage 4–5 males, we determined an NC cutoff point of 38 cm (sensitivity = 100%; specificity = 50%; PPV = 68.75%; and NPV = 100%).

We performed a binomial logistic regression analysis to determine the effects of ALT, gender, BMI *z score*, and NC on the likelihood of having NAFLD. We found this logistic regression model to be statistically significant [$X^2(4) = 31.72$, ($p < 0.0001$)]; it explained 39.5% of the variance in NAFLD (Nagelkerke's R^2), and it correctly classified the 75.8% of the patients. The model had a sensitivity of 81.25%, specificity of 69.77%, PPV of 75% and NPV of 76.92%. The only NAFLD predictive variables were NC (OR = 1.19; 95% CI = 1.02–1.40; p = 0.031) and ALT (OR = 1.07; 95% CI = 1.03–1.12; p = 0.001) (Table 2).

We conducted a correlation analysis of anthropometric and metabolic variables with NC and found that there were statistically significant correlations with WC (r = 0.812; p < 0.001), waist:height ratio (WHtR) (r = 0.268; p = 0.004), SBP (r = 0.543; p ≤ 0.001), ALT (r = 0.245; p = 0.019), AST:ALT ratio (r = −0.441; p ≤ 0.001), HOMA-IR (r = 0.417; p = 0.001), and insulin (r = 0.437; p < 0.001). The correlation between NC and elastography was significant (r = 0.529; p = 0.007) (Table 3).

Discussion

In this cross-sectional comparative study, we demonstrated that NC is a predicting factor for the diagnosis of NAFLD in pediatric populations with obesity, independent from levels of ALT, BMI *z score* value and gender. We found that there was a 19% greater risk of having NAFLD for every 1 cm increase in NC.

Previous studies have reported results that are consistent with our data. A Turkish study by Hatipoglu et al. found an association between NAFLD and NC in a pediatric population based on a multiple regression analysis–that is, that each 1 cm of NC increased the risk of NAFLD 1.544-fold in males and 1.733-fold in females [11].

Hatipoglu et al. reported an association between NC and NAFLD in both genders. In our study, we considered that the influence of pubertal stage is as important as the gender, particularly in our male population because gender can become a confounding factor due to the physiological growth of the cricoid cartilage observed in later Tanner stages.

We found clinical significance in Tanner stages 2–3 and Tanner stages 4–5 in our male population, but NC was not significant by NAFLD at ROC curves in our female population. These results match the findings of the study by Li et al., in which they only found clinically significant associations in their eutrophic adult male population [18].

In this study, we proposed the cutoff points predicting NAFLD in males in Tanner stages 2–3 as NC = 35 cm and in stages 4–5 as NC = 38 cm. Hatipoglu et al. suggested the use of cutoff points by Tanner stage; however, they did not consider the adjustment by gender we mentioned before, which could be an important confounding factor mainly in adolescent boys.

Another important finding in our study that strengthens the association between NC and NAFLD is the positive correlation between NC and the classical risk factors previously described for NAFLD in both genders, such as central adiposity, metabolic syndrome, hepatic enzyme alterations and insulin resistance [24–26].

This study found a positive correlation between NC and central adiposity parameters. Kelishadi et al. also reported an association between NC and overweight/obesity tested by central adiposity parameters (WC and WHtR) in a pediatric Iranian population (the CASPIAN IV study). This study also suggests that NC could be a useful screening tool to identify NAFLD in their population with overweight and obesity [12]. This relationship between NC and NAFLD in adults is unquestionable; however, the data in pediatric population is still considered scarce and inconsistent throughout studies, which highlights the importance of establishing cutoff points throughout ethnicities [11, 27].

Regarding the positive correlation that NC has with metabolic syndrome factors, we found a statistically significant association with WC and SBP. Several studies

Table 3: Neck circumference correlations with metabolic and anthropometric variables.

	WC	BMI *z score*	WHtR	SBP	Triglycerides	ALT	AST	AST/ALT ratio	GGT	Glucose	HOMA-IR	Insulin	Elastography	C-HDL
r	0.812	0.007	0.268	0.543	0.195	0.245	0.1	−0.441	0.414	0.109	0.417	0.437	0.529	−0.207
p-Value	**<0.001**	0.942	**0.004**	**<0.001**	0.057	**0.019**	0.355	**<0.001**	0.175	0.286	**0.001**	**<0.001**	**0.007**	0.051

r = Pearson correlation; $p < 0.05$ (significant values are in bold). WC, waist circumference; BMI, body mass index; WHtR, waist/height ratio; SBP, systolic blood pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase, GGT, gamma glutamyl transpeptidase; HOMA-IR, homeostasis model assessment insulin resistance index; C-HDL, high-density lipoprotein cholesterol.

have shown an association between NC and metabolic syndrome. For instance, Gomez-Albelaez et al., could establish this correlation and created NC cutoff points by gender in a Colombian pediatric population aged between 8 and 14 years. They could also find a correlation between NC with altered fasting glucose levels, low levels of HDL, triglycerides and blood pressure [16]. Kurtoglu et al. found similar results in a study performed in a pediatric population aged between 5 and 18 years [14]. Concerning high blood pressure, it is worth mentioning a case control study done in an adolescent population in Lithuania by Kuciene et al., in which they found that those patients with an NC above the 90th percentile adjusted by age and gender considerably elevated the risk of pre-hypertension (OR = 2.99 [1.88–4.77]) and hypertension (OR = 4.05 [3.03–5.41]) [13].

Through the analysis of metabolic alterations, we also found a correlation between insulin levels and HOMA-IR with NC. These outcomes resemble those reported in a study by Cassia da Silva et al., where they found this same relationship in a preadolescent male Brazilian population [28], which is consistent with findings reported by Gomez-Arbelaez et al. [16].

As we already know, ALT and AST are biochemical markers that have been universally used to assess liver damage in a wide variety of pediatric pathologies like NAFLD [29, 30]. In our study, only ALT and the AST/ALT ratio showed an association with NC. In previous studies in adults, this AST/ALT index has been associated, independently or as part of a score, with liver damage progression and severity as well as NASH [3, 31]. In pediatric populations, there has been few applications of this quotient, and previous studies were unable to establish a relationship with hepatic fibrosis grading, so we understand that conclusions about the screening for NAFLD in pediatrics with this marker is limited [32, 33].

We also found a correlation between the ARFI mean and NC. In our NAFLD group, the mean value was 1.88 m/s compared to the normal value in other studies (i.e. <1.19 m/s) [34]. A systematic review and meta-analysis also established that ARFI has an OR of 30.13, AUC of0.898, 80.2% sensibility and 85.2% specificity to diagnose hepatic fibrosis [35].

One of the limiting factors in our study was that we determined NAFLD diagnosis through hepatic ultrasound instead of a liver biopsy (gold standard) or magnetic resonance imaging (MRI) due to the high costs or invasiveness characteristics of these procedures. However, we considered that the information that was concluded in our study will become a useful contribution to the scarce existing information regarding the relationship between NAFLD and NC in the pediatric population. While the study by

Hatipoglu et al. could establish cutoff points for Tanner stage, we believe our results remain valuable to the Mexican pediatric population, particularly for boys, and that the results might have differed because of the ethnic background and the adjustment by gender and Tanner stage that we performed.

The NC is a novel anthropometric measure that has proven to be associated with different cardiometabolic risk factors. Its efficiency in most of the studies is like WC and therefore could have some advantages with respect to WC. It is an accessible method that only requires a tape measure, and the anatomic reference could be easier to learn and execute, allowing only one anthropometrist to perform the task. Also, for the patient, it may be more comfortable because it does not require undressing, which is a relevant issue for adolescents.

In our study, NC measurements have allowed us to propose cutoff points in our pubertal male population. These results are an interesting finding in our population considering that previous work from our group [36] analyzed other anthropometric and metabolic variables (HOMA-IR, WC, ALT, AST, visceral adiposity index and triglyceride levels) and found that these variables have associations with NAFLD only in the female population, suggesting that males have a higher risk of being underdiagnosed because of the lack of clinical or metabolic characteristics usually associated with this disease. The association of NC and NAFLD in the males of our study provides us with a tool to suspect NAFLD in this population.

Conclusions

We found an association between NC and NAFLD, in which an increase of 1 cm of NC was related to having 19% more risk of developing NAFLD. We could only establish NC cutoff points for male adolescents with Tanner staging 2–3 (NC = 35 cm) and Tanner staging 4–5 (NC = 38 cm). NC was also correlated with the already known risk factors for NAFLD, such as insulin resistance and central adiposity (WC and WHtR) as well as progression and severity risk factors like ARFI.

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SHORT COMMUNICATION

Serum levels of interleukin-1 beta associate better with severity of simple steatosis than liver function tests in morbidly obese patients

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Background: In high-fat diet-fed mice, interleukin-1 beta (IL-1 beta) has been shown to play a key role in hepatic steatosis. However, it remains unknown whether IL-1 beta could be associated with different grades of steatosis in obese humans. **Materials and Methods:** Morbidly obese patients (n = 124) aged 18–65 years were divided into four groups: no steatosis (controls), mild steatosis, moderate steatosis, and severe steatosis using abdominal ultrasound. IL-1 beta serum levels and liver function tests were measured and significant differences were estimated by one-way ANOVA followed by Tukey test. **Results:** IL-1 beta serum levels significantly increased in morbidly obese patients with mild (11.38 ± 2.40 pg/ml), moderate (16.72 ± 2.47 pg/ml), and severe steatosis (23.29 ± 5.2 pg/ml) as compared to controls (7.78 ± 2.26 pg/ml). Liver function tests did not significantly change among different grades of steatosis. **Conclusion:** IL-1 beta serum levels associate better with steatosis degree than liver function tests in morbidly obese population.

Key words: Fatty liver, interleukin-1 beta, liver functions tests, morbid, obesity

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INTRODUCTION

Simple steatosis is characterized by progressive fat accumulation in the liver and is considered to be the initial step in the development of nonalcoholic steatohepatitis (NASH), cirrhosis, and liver failure.[1,2] Liver function tests are typical biochemical markers to identify patients with NASH and cirrhosis, without showing utility in steatosis until now.[3]

Cytokines have been shown to play a key role in the pathogenesis and progression of steatosis in high-fat diet (HFD)-fed mice especially interleukin (IL)-1 beta (IL-1 beta).[4] However, as far as we know, there are no studies evaluating whether IL-1 beta could be modified depending on the simple steatosis degree in obese patients.[5]

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Our main goal was to determine the serum levels of IL-1 beta in morbidly obese patients with different degrees of simple steatosis, while also comparing the association level of IL-1 beta and liver function tests with severity of steatosis.

MATERIALS AND METHODS

Study population

Morbidly obese women and men (n = 124) aged 18–65 years, attending to the Department of Internal Medicine and the Clinic for Obesity of the General Hospital of Mexico from October 2014 to December 2017, were included in the study by convenience sampling and divided into four groups: controls (no steatosis), mild steatosis, moderate steatosis, and severe steatosis. Steatosis degree was scored by two independent senior hepatologists using

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abdominal ultrasound imaging, as previously described.[6] All participants provided written informed consent, and the study was conducted in strict adherence to the Helsinki Declaration. Exclusion criteria included previous diagnosis of acute or chronic hepatic disease, inflammatory or autoimmune disorders, acute or chronic infectious diseases, and neoplastic disorders, as well as pregnancy, lactation, and any kind of anti-inflammatory medical treatment. Blood samples were collected from each participant after 10–12 h fasting to avoid procedural variations.

Liver function tests and interleukin-1 beta serum levels

Liver function tests, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), and total bilirubin, were measured from serum samples by enzymatic assay (Megazyme International, Ireland). Blood glucose, insulin, HbA1c, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglycerides were also measured according to the manufacturer's instructions (Megazyme International, Ireland). IL-1 beta serum levels were measured by enzyme-linked immunosorbent assay (Peprotech, Mexico).

Statistical analysis

Data are expressed as mean ± standard deviation and were analyzed by one-way ANOVA followed by a *post hoc* Tukey test. ANOVA results were corrected using the Bonferroni's multiple comparison test. Women/men proportion in each group was evaluated using the Chi-square test. All statistical analyses were performed using the GraphPad Prism 6.01 software, GraphPad Software, La Jolla, CA 92037 USA. Differences were considered statistically significant when $P < 0.05$.

RESULTS

No significant differences were found in women/men proportion, age, body mass index, blood pressure, HDL, and triglycerides between control subjects (n = 27) and patients with mild (n = 35), moderate (n = 31), or severe steatosis (n = 31) [Table 1]. Severe steatosis was accompanied by significantly increased levels of blood glucose, insulin resistance, insulin, glycated hemoglobin, cholesterol, and LDL. Moderate steatosis was characterized by hyperinsulinemia resulting in low glucose values, even though insulin resistance was also increased in these patients. Mild steatosis was hallmarked by having similar levels of blood glucose, insulin, insulin resistance, cholesterol, and LDL than those found in morbidly obese patients without steatosis [Table 1].

All patient groups significantly differed from each other regarding IL-1 beta serum levels. As a matter of fact, IL-1 beta progressively elevated as the severity of steatosis also increased [Table 2]. Morbidly obese participants without steatosis (controls) showed 7.78 ± 2.26 pg/ml IL-1 beta in blood as compared to patients with mild, moderate, and severe steatosis, who exhibited 11.38 ± 2.40, 16.72 ± 2.47, and 23.29 ± 5.2 pg/ml IL-1 beta, respectively [Table 2]. Lower-upper 95% confidence intervals (95% CIs) for controls were 6.88–8.67 pg/ml IL-1 beta. On the contrary, lower-upper 95% CI for mild, moderate, and severe steatosis rose to 10.55–12.21, 15.81–17.63, and 21.34–25.23 pg/ml IL-1 beta, respectively. No significant differences were found in ALT, AST, GGT, ALP, and total bilirubin between control participants and patients with mild, moderate, or severe steatosis [Table 2].

Table 1: Demographic and biochemical characteristics of the study subjects

Parameters	Steatosis			
	Controls[a]	Mild[b]	Moderate[c]	Severe[d]
Sex (women/men)*	21/6	27/8	24/7	25/6
Age (years)	33.6±9.1	34.8±9.4	32.6±8.2	37.7±8.6
BMI (kg/m^2)	41.1±4.2	42.8±6.2	42.2±6.9	41.8±6.5
Blood pressure (mmHg)	132.86±13.10	131.86±13.9	131.86±12.10	131.87±14.11
Blood glucose (mg/dl)	102.4±11.6	103.5±18.1	96.7±12.7	132.9±23.6[a,b,c]
Serum insulin (mU/l)	14.4±5.7	14.2±5.3[c,d]	20.9±4.8[a,b]	21.2±5.2[a,b]
HOMA-IR	3.7±1.5	3.5±1.7[c,d]	5.1±1.3[b,d]	7.1±2.6[a,b,c]
HbA1C (%)	4.1±0.9	5.2±1.4[a,d]	5.4±0.8[a,d]	7.6±1.6[a,b,c]
Total cholesterol (mg/dl)	187.2±26.1	179.8±31.1[c,d]	208.5±39.4[b]	223.2±44.6[a,b]
LDL (mg/dl)	101.6±18.6	103.8±32.7[c,d]	136.8±24.3[a,b]	138.8±19.8[a,b]
HDL (mg/dl)	32.6±6.1	39.3±9.2	37.3±6.3	33.8±6.4
Triglycerides (mg/dl)	195.1±74.5	184.5±91.7	212.7±79.3	230.3±76.9

Data are expressed as mean±SD except for *that is expressed as absolute values. Significant differences were estimated by means of one-way ANOVA followed by a *post hoc* Tukey test except for *that was analyzed by means of the Chi-square test. ANOVA results were corrected using the Bonferroni's multiple comparisons test. Differences were considered significant when $P<0.05$, as follows: [a]Significant difference versus controls; [b]Significant difference versus mild steatosis; [c]Significant difference versus moderate steatosis; [d]Significant difference versus severe steatosis. BMI=Body mass index; HOMA-IR=Homeostatic model assessment of insulin resistance that resulted of multiplying glucose by insulin then divided by 22.5; HbA1c=Glycated hemoglobin; LDL=Low-density lipoprotein; HDL=High-density lipoprotein; SD=Standard deviation

Table 2: Serum levels of interleukin-1 β and liver function tests in morbidly obese patients with hepatic steatosis

Parameters	Steatosis			
	Controls[a]	Mild[b]	Moderate[c]	Severe[d]
IL-1β (pg/ml)	7.78±2.26	11.38±2.40[a,c,d]	16.72±2.47[a,b,d]	23.29±5.29[a,b,c]
ALT (U/L)	33.21±16.83	37.49±15.59	41.03±19.32	40.03±20.17
AST (U/L)	27.35±11.90	32.16±16.83	37.26±20.25	36.58±15.86
GGT (U/L)	28.87±16.41	32.51±16.26	32.78±18.69	37.89±21.60
ALP (U/L)	70.00±19.70	68.93±26.57	73.63±23.84	69.86±22.28
Total bilirubin (mg/dL)	0.78±0.26	0.73±0.31	0.84±0.39	0.93±0.30

IL-1β serum levels progressively increase according to the steatosis degree. ALT, AST, GGT, ALP, and total bilirubin do not significantly change among different grades of hepatic steatosis in the study population. Steatosis degree was scored by two independent senior hepatologists using abdominal ultrasound imaging. Data are expressed as mean±SD. Significant differences were estimated by means of one-way ANOVA followed by a *post hoc* Tukey test. ANOVA results were corrected using the Bonferroni's multiple comparisons test. Differences were considered significant when $P<0.05$, as follows: [a]Significant difference versus controls; [b]Significant difference versus mild steatosis; [c]Significant difference versus moderate steatosis; [d]Significant difference versus severe steatosis. IL=Interleukin; ALT=Alanine aminotransferase; AST=Aspartate aminotransferase; GGT=Gamma-glutamyl transpeptidase; ALP=Alkaline phosphatase; SD=Standard deviation

DISCUSSION

IL-1 beta is a member of the IL-1 cytokine family with prominent functions in the regulation of the inflammatory response as well cell proliferation, differentiation, and apoptosis.[7,8] However, IL-1 beta has now emerged as a major contributor in steatosis development by inducing hepatic lipogenesis and triglyceride overproduction in HFD-fed mice.[3,9] Consistent with previous information, our results demonstrate for the first time that IL-1 beta serum levels clearly increase in morbidly obese patients that show the most severe stages of steatosis, which in turn concurs with prior studies also reporting IL-1 beta elevation in portal and systemic blood of HFD-fed mice with hepatic steatosis.[10]

A growing body of studies have consistently reported increasing serum levels of tumor necrosis factor alpha, IL-6, IL-8, and IL-10 in NASH patients.[11,12] However, as far as we know, IL-1 beta had never been reported in morbidly obese patients with simple steatosis.[6,11,13] Interestingly, in morbidly obese participants without steatosis, serum IL-1 beta ranged from 2.02 to 11.63 pg/ml. On the contrary, in morbidly obese patients with mild, moderate, or severe steatosis, IL-1 beta levels were found between 4.87 and 19.07, 11.82 and 23.53, and 10.62 and 34.47 pg/ml, respectively, which suggest that serum values of this cytokine considerably increase only in severe grades of hepatic fat accumulation.

Finally, numerous studies have persistently shown that patients with simple steatosis exhibit normal liver function tests, except those who are progressing to NASH.[14,15] Our data expand on this body of work by revealing that liver function tests do not change in morbidly obese patients with different degrees of steatosis. In this sense, IL-1 beta appears to associate better with hepatic steatosis than liver function tests in patients with morbid obesity and should be further investigated in clinical prospective studies.

CONCLUSION

The serum levels of IL-1 beta associate better with severity of simple steatosis than liver function tests in morbidly obese population.

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Conflicts of interest

There are no conflicts of interest.

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Original article

Gender-specific differences in clinical and metabolic variables associated with NAFLD in a Mexican pediatric population

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ABSTRACT

Introduction and Objectives: Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in children and it is more prevalent in Hispanic males. The gender differences can be explained by body fat distribution, lifestyle, or sex hormone metabolism. We evaluated anthropometric and metabolic differences by gender in children with and without NAFLD.

Methods: We included 194 participants (eutrophic, overweight, and individuals with obesity). The presence of NAFLD was determined using ultrasonography, and we evaluated the association between this disease with metabolic and anthropometric variables by gender.

Results: The mean age was 10.64 ± 2.54 years. The frequency of NAFLD in boys was 24.51% and in girls was 11.96% (OR = 2.39; 95%CI = 1.10–5.19; $p = 0.025$). For girls, NAFLD was significantly associated with triglycerides ($p = 0.012$), homeostatic model assessment of insulin resistance (HOMA-IR) ($p = 0.048$), and the visceral adiposity index (VAI) ($p = 0.024$). The variables related to NAFLD in a gender-specific manner were body mass index (BMI) ($p = 0.001$), waist circumference (WC) ($p < 0.001$), HDL cholesterol ($p = 0.021$), alanine aminotransferase (ALT) ($p < 0.001$), and aspartate aminotransferase (AST) ($p = 0.002$).

Conclusions: In our study NAFLD is more frequent in boys, only ALT, and no other clinical or metabolic variables, were associated with NAFLD in these patients. HOMA-IR, VAI, triglyceride levels, and ALT were associated with NAFLD only in girls. The ALT cut-off points for the development of NAFLD in our study were 28.5 U/L in females and 27.5 U/L in males. Our findings showed that NAFLD should be intentionally screened in patients with obesity, particularly in boys.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined by the presence of steatosis in more than 5% of hepatocytes in patients with no history of alcohol consumption, no pharmacological treatment, and no congenital disease [1,2]. NAFLD is the most frequent type of liver disease in the pediatric population, with an overall prevalence of 7.6% and a cumulative prevalence of 34.2% in children with obesity [3]. Epidemiological studies of NAFLD in Mexico show a prevalence of 17.05% in asymptomatic adults and 12.6% in children with a body mass index (BMI) >85th percentile (overweight and obesity) [4,5].

Cross-sectional studies on humans and animal models have reported a higher prevalence of NAFLD in the male gender. In the pediatric population with obesity, the distribution by sex is 35.3% in males and 21.8% in females [3]. Central obesity, insulin resistance, dyslipidemia, Hispanic ethnicity, and the genetic variants COL13A1, ADIPOQ, SAMM50, and PNPLA3 in Mexican Americans

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and the Mexican-Mestizo population are the variables that are most frequently associated with NAFLD [6–9].

Although a higher prevalence of NAFLD has been reported in boys, gender-specific characteristics of this disorder have rarely been explored. It has been proposed that the gender-specific differences observed in the prevalence of NAFLD may result from the possible protective role of estrogens, which decrease the risk for this disorder among females. Recent findings on the physiopathology of NAFLD, evaluated from a gender-specific perspective, have indicated that lipid metabolism and body fat distribution may play a relevant role [10,11].

Pediatric patients are not frequently screened for NAFLD, and thus, it is rarely identified, primarily because of the complexity of the diagnosis [2,12]. This study aimed to evaluate the gender-specific differences in the clinical and metabolic variables in a pediatric population with NAFLD.

2. Methods

2.1. Study design

A cross-sectional comparative study was performed. Eutrophic, overweight, and individuals with obesity and ranging in age from 6–18 years were consecutively included from January 2015 to September 2016. Patients with overweight/obesity from the Childhood Obesity Clinic of the Hospital General de Mexico were invited to participate, whereas eutrophic children were recruited from surrounding schools. All patients included in this study were born and raised in Mexico City and the suburban area. The local ethics committee approved the protocol.

The participants were classified into the following groups, according to the Centers for Disease Control and Prevention guidelines: eutrophic (BMI <85th percentile); overweight (BMI ≥85th and <95th percentile); and obesity (BMI ≥95th percentile).

Patients with obesity that had a genetic or endocrine origin; those with a previously known liver disease, diabetes or other endocrinopathies; patients receiving a pharmacological treatment that was able to modify lipid or carbohydrate metabolism; and those who reported alcohol consumption were excluded.

Informed consent and assent were obtained from the participants and their parent/legal guardian. All sensitive patient data were protected in compliance with the Health Insurance Portability and Accountability Act (HIPAA). A complete medical record was documented for each participant.

The NAFLD diagnosis was determined by a liver ultrasound that was performed by an expert ultrasonographer.

2.2. Anthropometric and metabolic evaluation

The clinical variables assessed were age, gender, weight, height, BMI, waist circumference (WC), and sexual development stage.

A mechanical scale and a stadiometer were used to evaluate weight and height. The measuring location for WC was the midway point between the last costal cartilage and the anterosuperior iliac crest. Pediatricians and pediatric endocrinologists appraised the sexual status, according to the Tanner scale [13,14]. Arterial blood pressure was measured from the right brachial artery using a manual sphygmomanometer and special-sized pressure cuffs according to the patient's age, and the measurement was taken twice per patient, with the patient in a sitting position.

We calculated the BMI according to Quetelet's equation [15]. Additionally, using the homeostatic model assessment of insulin resistance (HOMA-IR), we calculated the insulin resistance index [16].

A gender-specific visceral adiposity index (VAI) was calculated using a formula that was previously proposed by our group for pediatric populations, as follows:

$$\text{Female VAI} = \text{WC}/(-0.018[\text{BMI}^2] + 3.3[\text{BMI}] + 7.86) \times (\text{Triglycerides}/0.91) \times (1.42/\text{HDL}); \quad \text{and}$$

$$\text{Male VAI} = \text{WC}/(-0.023[\text{BMI}^2] + 3.83[\text{BMI}] + 0.65) \times (\text{Triglycerides}/0.76) \times (1.37/\text{HDL}).$$

Metabolic variables were evaluated by obtaining venous blood after the patients had fasted for 12 h. Enzymatic methods were performed to measure glucose, aminotransferases, total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, and triglycerides, using available commercial kits. Insulin was determined through chemiluminescence analysis. Normal values were considered according to the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) and the Endocrine Society clinical practice guidelines for NAFLD and pediatric obesity, respectively (alanine aminotransferase (ALT) <25 U/L in boys and <22 U/L for girls, HDL <40 mg/dL, and triglycerides in patients 0–9 years <100 mg/dL and 10–19 years <130 mg/dL) [17,18].

2.3. Ultrasonography

The NAFLD diagnosis was performed using a B mode ultrasound and a 1–4 mHz curve transducer (Siemens Acuson S2000, Mountain View, CA, USA), according a standardized protocol, by an expert ultrasonographer who was blinded to the clinical and metabolic conditions [19,20]. The kappa coefficient for intra-observer concordance was 0.90. The steatosis condition was categorized as present/not present, according to the hepatic tissue's echogenicity, in contrast to the adjunct right kidney's parenchyma.

2.4. Statistical analyses

Descriptive and inferential statistics for numerical variables were reported as the mean and standard deviation (SD), and the percentages were used for categorical variables.

The inferential statistical analyses included a two-factor analysis of variance (ANOVA) for all anthropometric and metabolic variables, considering the presence of NAFLD and gender as factor variables, and we ensured that the test assumptions were met. The effect size was calculated using Cohen's *f* and was considered to be as follows: >0.10 small; >0.25 medium; and >0.40 large [21,22].

Chi-squared tests and Fisher's exact test were used to assess categorical variables. Binary logistic regression evaluated the association between NAFLD and BMI-Pc, ALT, aspartate aminotransferase (AST), WC by gender, and triglycerides by sex [23].

The most relevant variables related to NAFLD were evaluated using the receiver operating characteristics (ROC) curve analysis, according to gender. The cut-off points, sensitivity, and specificity were obtained for each variable [24,25].

All analyses were performed using the statistical software IBM SPSS Statistics version 22.0 (IBM, Armonk, NY, USA). A p value of <0.05 was considered to be statistically significant.

3. Results

3.1. Descriptive statistics

A total of 194 patients (102 males and 92 females) were recruited: 44 eutrophic, 38 overweight, and 112 with obesity. The mean age of the participants was 10.64 ± 2.54 years.

The overall NAFLD frequency in our study was 18.55%; it was 24.51% in boys and 11.96% in girls, which was significantly different (OR = 2.39; 95%CI = 1.10–5.19; $p = 0.025$).

In the obesity group, the frequency was 29.46%: 32.8% in males and 24.4% in females (OR = 1.51; 95%CI = 0.65–3.54; $p = 0.340$). We found that 91.7% of the patients with a NAFLD were obese.

The participants' demographic and anthropometric characteristics (by gender) are shown in Table 1.

3.2. Inferential statistics

Differences in anthropometric and metabolic variables by gender and the presence of NAFLD were assessed using two-factor ANOVAs.

We found statistically significant differences between gender and the presence of NAFLD for the following variables: triglycerides ($F(1,185) = 6.618$, $p = 0.012$, $f = 0.0895$); insulin ($F(1,174) = 6.197$, $p = 0.021$, $f = 0.087$); HOMA-IR ($F(1,174) = 4.28$, $p = 0.048$, $f = 0.0895$), and VAI ($F(1,185) = 5.47$, $p = 0.02$, $f = 0.0959$). The combined effect of the presence of NAFLD and gender was also evaluated for all significant ANOVAs. The mean triglyceride concentration in males with and without NAFLD and in females with and without NAFLD were 132.32 ± 68.14, 120.85 ± 68.08, 210.18 ± 73.26, and 126.38 ± 74.13 mg/dL, respectively. When comparing differences in triglyceride concentrations among the male and female patients with and without this disease, we found that the mean triglyceride concentration in female NAFLD patients was 77.86 mg/dL higher than that in males. Similarly, in the group without NAFLD, the triglyceride levels were higher in females compared to males, with a mean difference of 5.53 mg/dL. The difference between the differences was 70.993 (95%CI, 15.966–126.02, $p = 0.012$).

For insulin concentrations, the mean levels in males with and without NAFLD and in females with and without this disorder were 15.00 ± 5.69, 13.11 ± 6.75, 14.16 ± 12.08, and 14.92 ± 8.43 UI/L, respectively. In a mean comparison by gender and the presence of NAFLD, male NAFLD patients had higher insulin concentrations compared to females, with a mean difference of 0.84 UI/L, whereas among patients without NAFLD, females had higher insulin concentrations, with a mean difference of 1.81 UI/L. The difference between the differences was 7.357 (95%CI, 1.145–13.569, $p = 0.021$).

The mean HOMA-IR levels in males with and without NAFLD and in females with and without NAFLD were 3.24 ± 1.20, 2.56 ± 1.69, 5.20 ± 2.70, and 3.04 ± 1.99, respectively. In a mean comparison by gender and the presence of NAFLD, in patients with NAFLD, females had a higher mean HOMA-IR level compared to males, with a mean difference of 1.96 points. Similarly, in patients without NAFLD, the HOMA-IR level was also higher in females than in males, with a smaller mean difference of 0.48 points. The difference between the differences was 1.471 (95%CI, 0.013–2.928, $p = 0.048$).

For VAI scores, the mean values in males with and without NAFLD and in females with and without NAFLD were 2.87 ± 1.97, 2.57 ± 1.89, 4.30 ± 1.75, and 2.38 ± 1.66, respectively. In a mean comparison by gender and the presence of NAFLD, in NAFLD patients, the VAI scores were higher in females compared to males, with a mean difference of 1.43 points. Similarly, among the participants without NAFLD, females had higher VAI scores than males, with a mean difference of 0.19 points. The difference between the differences was 1.62 (95%CI, 0.215–3.024, $p = 0.024$) (Table 1 and Fig. 1).

For the variables that did not demonstrate significant interactions in factorial ANOVAs, the main effects were evaluated. The following significant main effects from NAFLD were observed: for BMI-Percentile (Pc) the mean was 97.48 ± 2.01 for patients with NAFLD *vs.* 83.87 ± 22.52 for those without NAFLD ($p = 0.001$); for WC, the mean was 86.82 ± 12.73 with NAFLD *vs.* 77.43 ± 13.47 without NAFLD ($p < 0.001$); for HDL cholesterol, the mean was 37.53 ± 6.93 with NAFLD *vs.* 41.54 ± 10.21 without NAFLD ($p = 0.018$); for ALT, the mean was 48.28 ± 21.98 with NAFLD *vs.* 22.22 ± 9.81 without NAFLD ($p < 0.001$); and for AST, the mean was 36.14 ± 10.55 with NAFLD *vs.* 27.22 ± 6.58 without NAFLD ($p = 0.002$; Table 1 and Fig. 1).

A binomial logistic regression was performed to ascertain the effects of BMI percentile, ALT, WC by gender, and triglycerides by gender on the likelihood that participants would have NAFLD. The logistic regression model was statistically significant ($\chi^2(6) = 79.47$, $p < 0.0001$). The model explained 54.9% (Nagelkerke's R^2) of the variance in NAFLD and correctly classified 81.2% of the cases. The sensitivity of the model was 79%, and specificity was 90%; the positive predictive value was 53%, and the negative predictive value was 97%. The model was then internally validated using the ROC curve. The AUC value was 0.922 (95%CI = 0.877–0.966, $p < 0.0001$). The only statistically significant variable was ALT (Table 2).

With the aim of evaluating the accuracy of the variables associated with NAFLD by gender, we performed receiver-operating characteristics (ROC) curve discrimination. For female participants

Table 1
Clinical and metabolic variables associated to NAFLD by gender in Mexican children.

Variables	Male		Female		Gender	NAFLD	Gender and NAFLD
	NAFLD $n = 25$	No NAFLD $n = 77$	NAFLD $n = 11$	No NAFLD $n = 81$	p	p	p
Age (years)	11.08 ± 2.04	10.26 ± 2.47	10.83 ± 3.27	10.85 ± 2.64	0.736	0.423	0.406
BMI (percentile)	97.33 ± 2.19	85.85 ± 22.49	97.82 ± 1.56	81.72 ± 23.75	0.661	**0.001**	0.579
SBP (mmHg)	99.17 ± 8.66	96.59 ± 8.92	100.91 ± 9.44	96.54 ± 8.83	0.493	0.083	0.461
DBP (mmHg)	63.75 ± 6.25	62.03 ± 6.72	64.09 ± 7.36	61.91 ± 6.92	0.925	0.171	0.781
WC (cm)	86.82 ± 12.73	77.57 ± 13.66	86.46 ± 14.51	75.89 ± 11.89	0.670	**<0.001**	0.388
Glucose (mg/dL)	88.00 ± 5.88	88.38 ± 5.83	86.73 ± 7.17	86.23 ± 11.59	0.325	0.972	0.800
Triglycerides (mg/dL)	132.32 ± 68.14	120.85 ± 68.08	210.18 ± 73.26	126.38 ± 74.13	**0.003***	**0.001***	**0.012**
Total-C. (mg/dL)	158.64 ± 26.64	163.62 ± 31.14	171.27 ± 30.24	154.86 ± 30.59	0.747	0.342	0.076
HDL-C (mg/dL)	38.40 ± 6.72	42.61 ± 12.76	35.55 ± 7.30	40.78 ± 8.54	0.247	**0.021**	0.801
ALT (IU/L)	49.0 ± 24.50	24.49 ± 10.94	46.64 ± 15.67	22.50 ± 24.57	0.173	**<0.001****	0.662
AST (IU/L)	36.16 ± 11.41	28.83 ± 7.10	36.09 ± 8.80	27.43 ± 16.66	0.768	**0.002***	0.789
Insulin (IU/L)	15.00 ± 5.69	13.11 ± 6.75	14.16 ± 12.08	14.92 ± 8.43	**0.001***	**0.001***	**0.021**
HOMA-IR	3.24 ± 1.20	2.56 ± 1.69	5.20 ± 2.70	3.04 ± 1.99	**0.001***	**<0.001***	**0.048**
VAI	2.87 ± 1.97	2.57 ± 1.89	4.30 ± 1.75	2.38 ± 1.66	0.083	**0.002***	**0.024**

Values expressed as means ± standard deviation.
NAFLD: non-alcoholic fatty liver disease; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; WC: waist circumference; C: cholesterol; ALT: alanine aminotransferase; AST: aspartate aminotransferase; HOMA-IR: homeostatic model assessment; VAI: visceral adiposity index.
Effect size (f) = *0.10–0.24; **0.25–0.39.
Bold values are p values < 0.05.

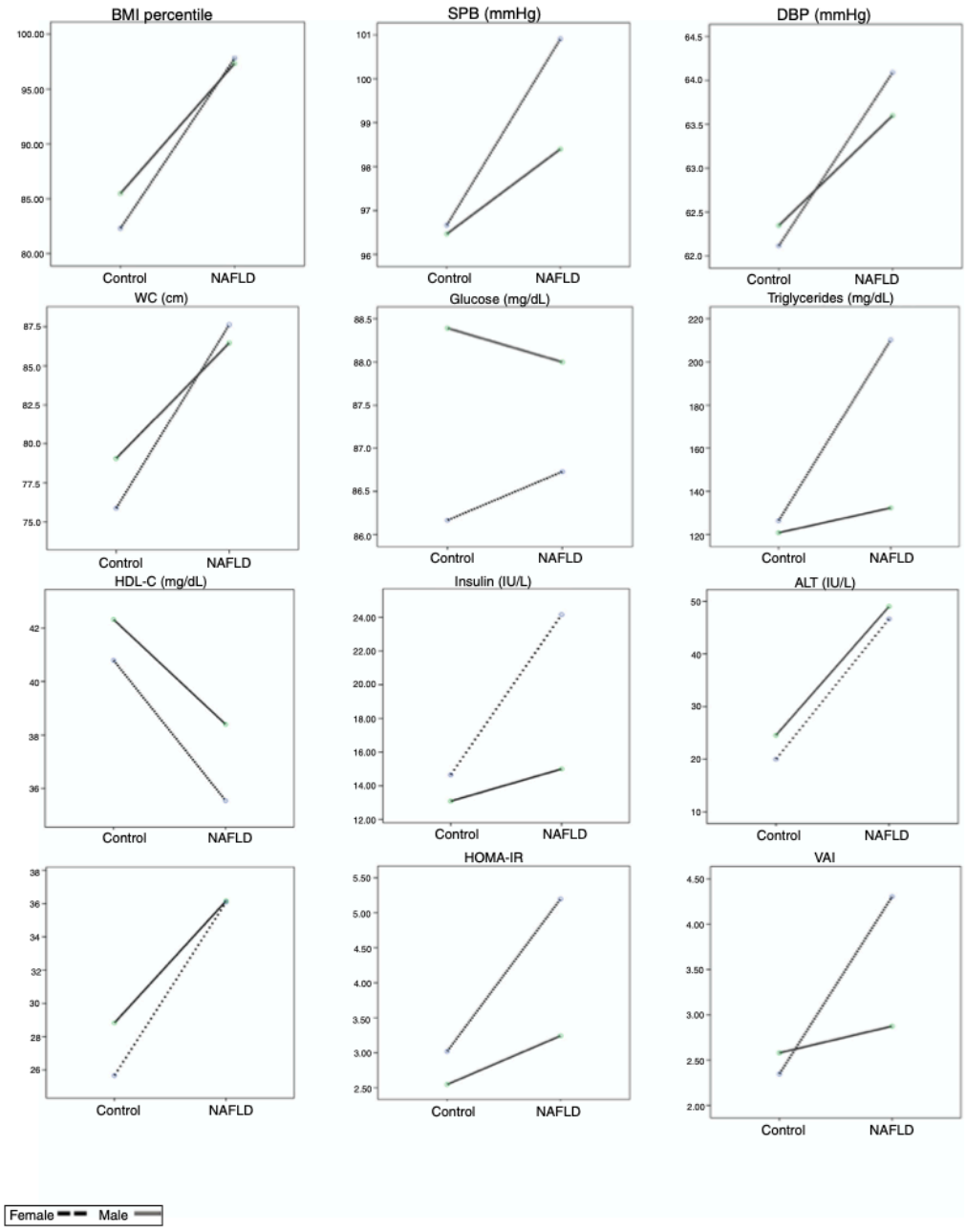


Fig. 1. Clinical and metabolic variables adjusted by gender and NAFLD condition in Mexican children.
On *X* axis NAFLD condition is expressed. The values on *Y* axis express the estimated marginal means.
NAFLD: non-alcoholic fatty liver disease; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; WC: waist circumference; Chol.: cholesterol; ALT: alanine aminotransferase; AST: aspartate aminotransferase; HOMA-IR: homeostatic model assessment – insulin resistance; VAI: visceral adiposity index.
Female: ▬ ▬ ; Male: ▬▬ .

with NAFLD, we found a significant AUC value for ALT, AST, WC, HOMA-IR, triglycerides, and VAI values. Based on these results, and by calculating the Youden index, we obtained the cut-off points for these variables in males and females. In females, the optimal cut-off points were as follows: ALT, 28.5 U/L (sensitivity 100%, specificity 89.2%); AST, 27.5 U/L (sensitivity 90.90%, specificity 62.20%); WC, 79.5 cm (sensitivity 90.90%, specificity 64.90%); HOMA-IR, 2.08 (sensitivity 100%, specificity 44.6%); triglycerides, 128 mg/dL (sensitivity 81.8%, specificity 69%); and VAI score, 2.33 (sensitivity 100%, specificity 63.5%).

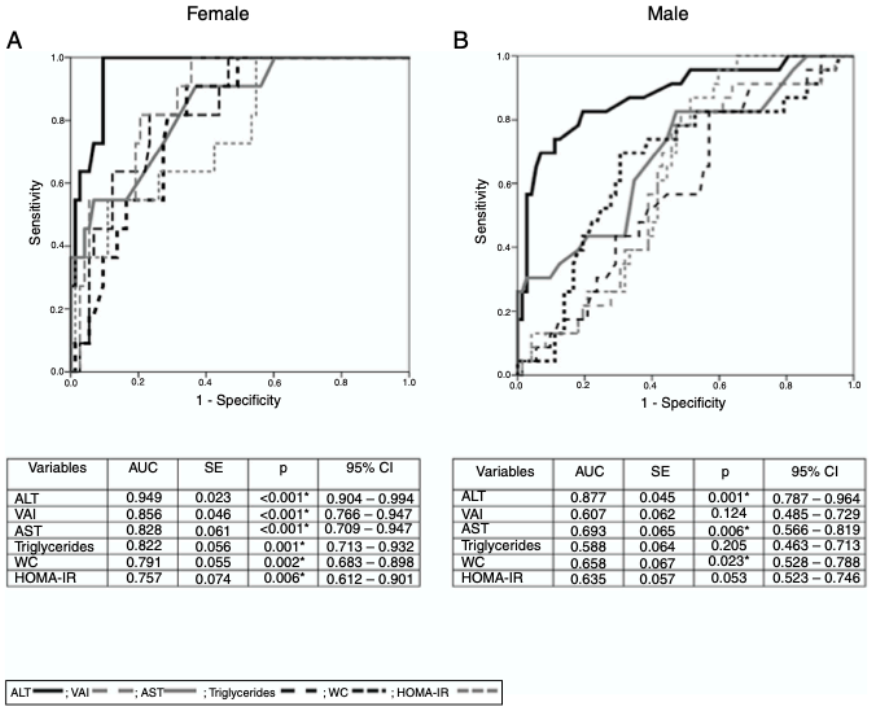
In male patients, the only significant AUC value was found for ALT concentrations, and the cut-off point was 27.5 U/L (sensitivity 82.60%, specificity 80.60%; Fig. 2).

The presence of metabolic syndrome (MS) in females with and without NAFLD was statistically significant, as shown by Fisher's exact test (OR = 9.019; 95%CI = 2.30–35.30; $p = 0.002$). However, in

Table 2
Logistic regression predicting likelihood of NAFLD in Mexican children based on BMI, ALT, WC by gender and triglycerides by gender.

Variable	*B*	SE	*p*	OR	95%CI	
					Lower bound	Upper bound
BMI (percentile)	0.148	0.094	0.116	1.159	0.964	1.394
ALT (IU/L)	0.100	0.020	**<0.001**	1.106	1.062	1.151
WC by gender	0.014	0.013	0.281	1.014	0.988	1.041
Triglycerides by gender	−0.008	0.006	0.230	0.992	0.980	1.005

NAFLD: non-alcoholic fatty liver disease; BMI: body mass index; ALT: alanine aminotransferase; AST: aspartate aminotransferase; WC: waist circumference; SE: standard error; OR: Odds Ratio; CI: confidence interval. Bold values are p values < 0.05.



Variables	AUC	SE	p	95% CI
ALT	0.949	0.023	<0.001*	0.904 – 0.994
VAI	0.856	0.046	<0.001*	0.766 – 0.947
AST	0.828	0.061	<0.001*	0.709 – 0.947
Triglycerides	0.822	0.056	0.001*	0.713 – 0.932
WC	0.791	0.055	0.002*	0.683 – 0.898
HOMA-IR	0.757	0.074	0.006*	0.612 – 0.901

Variables	AUC	SE	p	95% CI
ALT	0.877	0.045	0.001*	0.787 – 0.964
VAI	0.607	0.062	0.124	0.485 – 0.729
AST	0.693	0.065	0.006*	0.566 – 0.819
Triglycerides	0.588	0.064	0.205	0.463 – 0.713
WC	0.658	0.067	0.023*	0.528 – 0.788
HOMA-IR	0.635	0.057	0.053	0.523 – 0.746

Fig. 2. ROC curves for NAFLD by gender and ALT, AST, WC, VAI, HOMA-IR and triglycerides.
(A) Figure and table for female ROC curve. (B) Figure and table for male ROC curve.
NAFLD: non-alcoholic fatty liver disease; ALT: alanine aminotransferase; AST: aspartate aminotransferase; WC: waist circumference; VAI: visceral adiposity index; HOMA-IR: homeostatic model assessment – insulin resistance; AUC: area under curve; SE: standard error; CI: confidence interval; *$p < 0.05$.
ALT: ▬ ; VAI: ▬ ▬ ; AST: ▬ ; triglycerides: ▬ ▬ ; WC: ▬ ▬ • ; HOMA-IR: ▬ ▬ .

males with and without NAFLD, there was no significant difference (OR = 1.125; 95%CI = 0.36–3.51; $p = 0.84$).

4. Discussion

Our study results revealed gender-specific differences in metabolic and anthropometric variables related to NAFLD. Moreover, we found evidence that the variables that were positively associated with insulin resistance, such as insulinemia, HOMA-IR, triglyceride concentration, and VAI scores, were also associated with NAFLD in a gender-specific manner. For example, VAI, HOMA-IR, and triglycerides were significantly higher in female patients with NAFLD compared to males. However, in the regression analysis, the only variable that significantly predicted the presence of NAFLD was ALT, which increased the risk of NAFLD by 10% for each unit increment for both genders.

Mexico currently has one of the highest proportions people with obesity, and consistent with the prevalence of NAFLD, the frequency of NAFLD in our obesity group was 29.46%. The development of NAFLD requires a complex interaction of multifactorial features such as genetic susceptibility and environmental factors. Ramos-López et al. analyzed the pattern of dietary intake in a Mexican population with and without liver disease, and they found that both have a diet high in fat and cholesterol that lacks polyunsaturated fatty acids and some micronutrients with antioxidant properties that have been associated with the development of liver damage in the general population. The previous statements could explain the relationship between pediatric patients and the development of NAFLD through the influence of genetic variants of risk, a sedentary lifestyle, and the Mexican obesogenic diet [26].

In our study, the frequency of NAFLD by gender was higher in boys compared to girls, with a 2:1 ratio. Previous studies that were included in an epidemiological meta-analysis of NAFLD showed results similar to ours. Although the diagnostic methodologies used in the studies included in this meta-analysis were heterogeneous, the most frequently used method for diagnosing NAFLD was ultrasonography [3,11,27].

NAFLD is associated with various anthropometric and metabolic factors, such as high BMI, ethnic group (with a higher prevalence

in Asian and Hispanic patients), male gender, insulin resistance, and dyslipidemia. BMI has also been related to increased visceral and profound subcutaneous adiposity, which are also considered to be risk factors for NAFLD [28]. In our study, univariate analyses performed by gender and the presence of NAFLD showed that in males, the only variables that were significantly related to NAFLD were ALT and those that indirectly reflect visceral adiposity (BMI and WC). However, in females, NAFLD was also associated with a higher BMI, WC, and ALT, as well as higher insulin, HOMA-IR, and VAI. Although in previous studies the variables involved in NAFLD and insulin resistance are frequently reported, we found that there is a gender-specific association between these variables and the female population [2,6,7].

Our results also showed a significant association between NAFLD and the BMI-Pc, WC, HDL-cholesterol, and ALT concentration, with no differences observed between the genders. This finding is in accordance with that of Lonardo et al. and Singh et al., who reported that BMI, WC, waist-to-height ratio, and waist-to-hip ratio are gender-independent predicting factors for NAFLD in adults [29,30].

ROC curve discrimination analysis by gender showed a significant association between NAFLD and AST, ALT, triglycerides, HOMA-IR, insulin, and VAI only in girls. In boys, the only variable that behaved as a possible diagnostic biomarker was ALT, confirming the gender-specific behavior. The means for ALT in both genders from our study are aligned with the cut-off points that are recommended by the SAFETY study and the Council for the Treatment of Child Obesity published by the Endocrine Society [18,31].

In the last two decades, researchers worldwide have described NAFLD as a condition with sexual dimorphism. Given the complex multifactorial pathophysiology of NAFLD, research on the sexual dimorphism inherent to this disease may lead us to a better understanding of the pathophysiological processes that are involved [11,29].

Women tend to have more subcutaneous adipose tissue and higher leptin levels, which, combined with estrogen production, protects them from the accumulation of fatty tissue in the visceral compartment. In contrast, the distribution of adipose tissue in males is primarily visceral, a condition that is associated with insulin resistance and a greater influx of free fatty acids into the portal venous system, thereby promoting NAFLD [10,11,32]. In our study, we did not observe an association between NAFLD and any of the factors related to insulin resistance in boys.

The influence of sex steroids on metabolic disturbances remains an unanswered question because hyperandrogenic women as well as men with androgen deficiency tend to develop abdominal obesity, insulin resistance, type 2 diabetes, and NAFLD, and they have a higher cardiovascular risk [33].

Moreover, women with polycystic ovarian syndrome (PCOS) have an increased prevalence of NAFLD compared to age and BMI-paired controls (OR = 2.54; 95%CI = 2.19–2.95), and NAFLD-PCOS women have an androgen bioavailability that increases independently of BMI and insulin resistance [34–36]. Conversely, androgen deficiency in men has been related to NAFLD, independent of other known risk factors such as insulin resistance and visceral adiposity [37]. These studies show that female androgen excess and male androgen deficiency have similar metabolic phenotypes, showing the complexity of androgens' role in metabolism.

The variables in our study that were associated with NAFLD in females are consistent with those observed in previous reports, and these include insulin resistance, hypertriglyceridemia, and visceral adiposity (evaluated as VAI and WC in our study). Given that we did not find an association between these variables and NAFLD in males, we believe that it is possible that the pathophysiological mechanisms in this population may differ from those involved in insulin resistance.

For lipid metabolism, studies performed in humans and in animal models have shown an increased ability in females to metabolize free fatty acids by lipolysis. However, males can have prolonged *de novo* lipogenesis, which may condition them for a longer period of free fatty acid exposure and their associated damage. These changes could help us to understand why men are more likely to develop NAFLD [38].

A study performed in the United Kingdom evaluated fatty acid metabolism according to gender, and it showed that the beta-oxidation during fasting in men is less efficient compared to women, and it also has longer lasting *de novo* lipogenesis. Both phenomena may promote esterification and storage of free fatty acids in the liver, and consequently, stimulate the development of NAFLD in the male population [38].

Experimental models have often shown that androgens and estrogens are balanced in the modulation of hepatic lipid metabolism. Zhang et al. suggested that the combined administration of selective androgen and estrogen receptor modulators could be a potential treatment for patients with NAFLD [39]. Considering that there is a fluctuation of estrogens or androgens in teenagers, we attempted to evaluate the effect of these hormones by pubertal stage; however, there were no differences between pubertal and prepubertal patients.

The Metabolic syndrome (MS) is a critical factor that is related to NAFLD, to the extent that NAFLD has been considered by many to be the hepatic manifestation of this syndrome [40,41]. Concerning the association of this disorder with MS, it is relevant to say that in our study, a statistically significant relationship was observed between NAFLD and MS in females but not in males. The frequency of NAFLD in patients with fewer than three criteria for MS in our study was 63.9%. These results are clinically relevant, considering that in children with obesity, the presence of MS is believed to be the most important risk factor for the development of other comorbidities [42,43]. Our results contribute to the evidence that supports considering each component of the MS as a risk factor for NAFLD, particularly hypertriglyceridemia (>110 mg/dL) [44]. Srinivas et al. studied, from a gender perspective, the association of NAFLD with MS component risk factors in an urban cohort, and they found that the components related to this disorder in women were hyperglycemia and hypertriglyceridemia, whereas in men, only BMI was related (used as a criterion for MS instead of WC) [27]. In our study, the only risk component among males was the indirect measure of adiposity (WC), while in females, it was low HDL levels and hypertriglyceridemia.

This study has some limitations. The most relevant limitation is that the diagnosis of NAFLD was not performed using the gold standard (liver biopsy), because of the inherent risks related to the procedure and ethical issues. We evaluated NAFLD using ultrasonography because of accessibility in our population.

According to our study, ALT, AST, WC, triglycerides, HOMA-IR, and VAI were only related to NAFLD in the female population. Logistic regression showed that only ALT was predictive of NAFLD in both genders. The ALT cut-off points for the development of NAFLD in our study were 28.5 U/L (sensitivity 100%, specificity 89.2%) in females and 27.5 U/L (82.60% sensitivity, 80.60% specificity) in males. Although ALT was the only predictor for NAFLD in boys, it does not have the same diagnostic efficiency as it does in females.

5. Conclusions

We demonstrated that, although NAFLD is more frequent in boys, only ALT, and no other clinical or metabolic variables, were associated with NAFLD in these patients. HOMA-IR, VAI, triglycerides levels, and ALT were associated with NAFLD only in girls.

The ALT cut-off points for the development of NAFLD in our study were 28.5 U/L in females and 27.5 U/L in males. Our results suggest that we may consider each MS criterion individually as a risk factor for developing NAFLD. Our findings showed that NAFLD should be strongly suspected and that patients with obesity should be intentionally screened for NAFLD, particularly boys.

Further research is required using non-invasive methods and biomarkers that can help us to predict NAFLD in children and teenagers.

Abbreviations

NAFLD	non-alcoholic fatty liver disease
HOMA-IR	homeostatic model assessment of insulin resistance
VAI	visceral adiposity index
BMI	body mass index
WC	waist circumference
HDL	high density lipoprotein
ALT	alanine aminotransferase
AST	aspartate aminotransferase
HIPAA	Health Insurance Portability and Accountability Act
LDL	low density lipoprotein
SD	standard deviation
ANOVA	analysis of variance
ROC	receiver operating characteristics
AUC	area under curve
PCOS	polycystic ovarian syndrome
FAI	free androgen index

Ethics

Informed consent and assent were obtained from the participants and their parent/legal guardian. All sensitive patient data were protected in compliance with the Health Insurance Portability and Accountability Act (HIPAA).

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Author contributions

VOE and GNN conceived the idea and evaluate the patients.

VOE, GNN, LAJC and EG contributed to the interpretation of the results.

GHMJ, QG and CCS contribute to patients recruitment and collected the data.

HRA performed the ultrasonographic evaluation.

LAJC and LSE performed the statistical analysis of the data.

All authors discussed the results and contribute to the final manuscript.

Conflict of interest

The authors have no conflicts of interest to declare.

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Effects of Conjugated Linoleic Acid and Metformin on Insulin Sensitivity in Obese Children: Randomized Clinical Trial

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Context: Insulin resistance precedes metabolic syndrome abnormalities and may promote cardiovascular disease and type 2 diabetes in children with obesity. Results of lifestyle modification programs have been discouraging, and the use of adjuvant strategies has been necessary.

Objective: This study aimed to evaluate the effects of metformin and conjugated linoleic acid (CLA) on insulin sensitivity, measured via euglycemic-hyperinsulinemic clamp technique and insulin pathway expression molecules in muscle biopsies of children with obesity.

Design: A randomized, double-blinded, placebo-controlled clinical trial was conducted.

Setting: Children with obesity were randomly assigned to receive metformin, CLA, or placebo.

Results: Intervention had a positive effect in all groups. For insulin sensitivity Rd value (mg/kg/min), there was a statistically significant difference between the CLA vs placebo (6.53 ± 2.54 vs 5.05 ± 1.46, $P = 0.035$). Insulinemia and homeostatic model assessment of insulin resistance significantly improved in the CLA group ($P = 0.045$). After analysis of covariance was performed and the influence of body mass index, age, Tanner stage, prescribed diet, and fitness achievement was controlled, a clinically relevant effect size on insulin sensitivity remained evident in the CLA group (37%) and exceeded lifestyle program benefits. Moreover, upregulated expression of the insulin receptor substrate 2 was evident in muscle biopsies of the CLA group.

Conclusions: Improvement of insulin sensitivity, measured via euglycemic-hyperinsulinemic clamp and IRS2 upregulation, favored patients treated with CLA. (***J Clin Endocrinol Metab*** **102: 132–140, 2017)**

Obesity is a multifactorial disease with high prevalence in Mexico. According to the 2012 National Survey of Health and Nutrition, the prevalence of overweight and obese children and adolescents in Mexico is 34% (1). Insulin resistance has been recognized as the main physiopathological event preceding

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Abbreviations: ANCOVA, analysis of covariance; BMI, body mass index; CLA, conjugated linoleic acid; EHCT, euglycemic-hyperinsulinemic clamp technique; GLUT-4, glucose transporter 4; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; IRS, insulin receptor substrate; LIP, lifestyle intervention program; MET, metformin; PLB, placebo; QUICKI, quantitative insulin sensitivity check index; Rd, rate of glucose disposal; SEM, standard error of the mean.

metabolic syndrome abnormalities and may promote cardiovascular disease and type 2 diabetes in individuals with obesity (2). Lifestyle modification through healthy food selection and consumption, a regular physical activity program, and optimal sleep hygiene have been proposed as the gold standard of care in these individuals. Unfortunately, the compliance and success of these strategies are usually disappointing (3, 4), making pharmacological approaches somewhat necessary. Metformin (MET) is a biguanide used for the treatment of type 2 diabetes in children and adolescents due to its ability to decrease hepatic glucose production and increase peripheral insulin sensitivity. MET has been proposed as an adjuvant treatment in pediatric obesity efforts, especially in the presence of insulin resistance and its comorbidities. MET has beneficial effects on weight reduction and insulin resistance in obese nondiabetic individuals (5, 6).

Conjugated linoleic acid (CLA) is a group of isomers of linoleic acid, which are synthesized in the cud of ruminant animals by fermentative bacteria (7). CLA is present in dairy products, meat, and fat from beef and lamb. The most common CLA isomer contained in these products is *cis*-9,*trans*-11, which can be commercially synthesized from linoleic acid–rich oils and prepared as a 50% mixture with the *trans*-10,*cis*-12 isomer (8). Several studies have acknowledged the beneficial effects of CLA isomers on body composition (9, 10), immune response (11), bacterial-induced colonic inflammation (12, 13), as well as improvements in insulin sensitivity and lipid metabolism in experimental animals and humans (9). Additionally, CLA purportedly reduces fatty acid synthesis in adipocytes, suggesting that this supplement decreases fat deposition, directly contributing to an improvement in body composition in adults and children (14). Nonetheless, the impact of CLA on human health and disease is still controversial and research on this matter continues.

Based on the current obesity frequency in Mexico, and considering the limited and discouraging outcomes of intervention programs, adjuvant strategies must be installed. The objective of the present study was to evaluate the effects of MET and CLA on insulin sensitivity, measured via the euglycemic-hyperinsulinemic clamp technique (EHCT), in children with obesity.

Subjects and Methods

We performed a randomized, double-blinded, 16-week placebo (PLB)-controlled trial in the Pediatric Obesity Clinic at the Pediatrics Department of Hospital General de México (Mexico City, Mexico).

Patients with obesity aged 8 to 18 years who had not been previously intervened and had optimal psychological health were included in the study. Obesity was defined using Centers for Disease Control and Prevention criteria [body mass index (BMI) ≥ 95th percentile]. Exclusion criteria included BMI ≥ 35 kg/m^2, genetic or endocrine obesity, a systemic illness, diabetes or prediabetes (according to American Diabetes Association criteria) (15), and the use of weight loss medications that could modify lipids and glucose concentrations. The study (no. DI/11/311/04/108) was approved by the hospital's institutional review board; additionally, it was registered in ClinicalTrials.gov (no. NCT02063802).

All participants were included in the standardized healthy lifestyle program addressed to children and their parents. This 4-month program consisted of a monthly visit that included a 1-hour structured physical activity session (coordinated by a physical trainer), followed by a psychoeducational group session. The following information was presented to all participants: (a) description of a balanced and healthy nutrition, (b) emotion-related eating behavior and family support, (c) the benefits of physical activity, and (d) obesity-related comorbidities. These sessions were coordinated by nutritionists, psychologists, pediatricians, pediatric endocrinologists, and a physical trainer. Afterward, all patients held a medical consultation to evaluate their anthropometry and medical condition, as well as their progression and acquisition of skills and their compliance to the program. At the beginning of the intervention a complete nutritional evaluation was performed and a diet based on age, pubertal stage, and physical activity requirements, according to the World Health Organization and Food and Agricultural Organization guidelines, was prescribed (16). The recommended diet composition was 55% carbohydrates, 20% proteins, 25% lipids (<7% saturated fat, <300 mg/d cholesterol, and <1% *trans* fat), and <3 g of salt per day. Participants filled out a 24-hour nutritional recall questionnaire during the 3 days prior to their follow-up appointment to assess diet compliance. All patients were encouraged to participate in sports activities at last 5 days a week and for a minimum of 60 minutes.

To evaluate physical activity compliance, we tested fitness achievement using the Harvard step test modified for the pediatric population and a physical fitness score was calculated (17); evaluations were applied at baseline and at the postintervention state. The overall intervention compliance was evaluated through anthropometric, metabolic, and fitness parameter modifications, as well as through the acquisition of healthy behavior knowledge.

Clinical trial design

This trial was conducted in accordance with the Declaration of Helsinki and adhered to Good Clinical Practice Guidelines issued by the International Conference of Harmonization. The children and their parents provided written informed assent/consent. Eligible patients were included in the lifestyle intervention program (LIP) and randomized to receive either MET (1 g/d), CLA containing 50:50 isomers *c9,t11* and *t10,c12* (3 g/d), or PLB (1 g/d) 3 times a day for 16 weeks. Visits were scheduled monthly. Diet, exercise, and medication compliance, as well as anthropometric variables, were recorded during each visit. The final evaluation was similar to baseline; EHCT and skeletal muscle biopsies were performed at the postintervention state. Patients were eliminated when they showed poor compliance to medication (<80% or >100%) or intolerance, or when ≥1 workshop sessions were missed.

Anthropometric and metabolic evaluation

Baseline evaluation consisted of complete anthropometric and body composition analysis. Height and weight were obtained

defined as prepubertal; Tanner stages 2 to 3, defined as early puberty; and Tanner stages 4 to 5, defined as late puberty) was assessed after a clinical inspection of the mammary glands, testes volume, and pubic hair. Demographic and baseline characteristics were similar among the groups (Table 1).

Anthropometric and metabolic results

No significant differences were observed in baseline anthropometric and metabolic parameters or insulin resistance measured by surrogate indexes of insulin resistance (fasting insulinemia, HOMA-IR, and QUICKI). Distribution of Tanner stage status did not differ among the groups (χ^2 test, $P = 0.415$).

The overall impact of the intervention showed a positive effect on weight, height, BMI, and waist circumference, as well as on surrogated indexes of insulin resistance and physical fitness score in all of the groups (Table 2). No statistically significant differences were observed in these parameters between treatment groups. No differences were evident when comparing surrogate indices of insulin resistance during the postintervention phase among the groups.

Insulin sensitivity measured by EHCT

The primary outcome, insulin sensitivity, calculated as the Rd value, showed significant difference between the CLA group compared with PLB (6.53 ± 2.54 vs 5.05 ± 1.46, $P = 0.035$, Cohen's d effect size of 74%) (Table 3). Moreover, fasting insulinemia (Fig. 2) and HOMA-IR (Fig. 3) significantly decreased in the CLA group ($P = 0.04$). The adjusted analysis for controlling the influence of modifying or confounding variables such as BMI, change in BMI, age, Tanner stage, as well as dietary and physical program compliance, over the Rd value, showed that the Tanner stage had an independent effect over the Rd value ($P < 0.001$). When ANCOVA was executed and the aforementioned variables were controlled, no statistically significant differences were found between the three groups with regards to Rd value. Nonetheless, a clinically relevant effect size remained evident when comparing the CLA and PLB groups (Cohen's d effect size of 37%), suggesting a decrease in insulin resistance in patients receiving CLA. The effect size of MET vs PLB was 10% (5.72 ± 3.1 vs 5.38 ± 3) and that of MET vs CLA was 20% (5.72 ± 3.1 vs 6.34 ± 2.8), favoring the

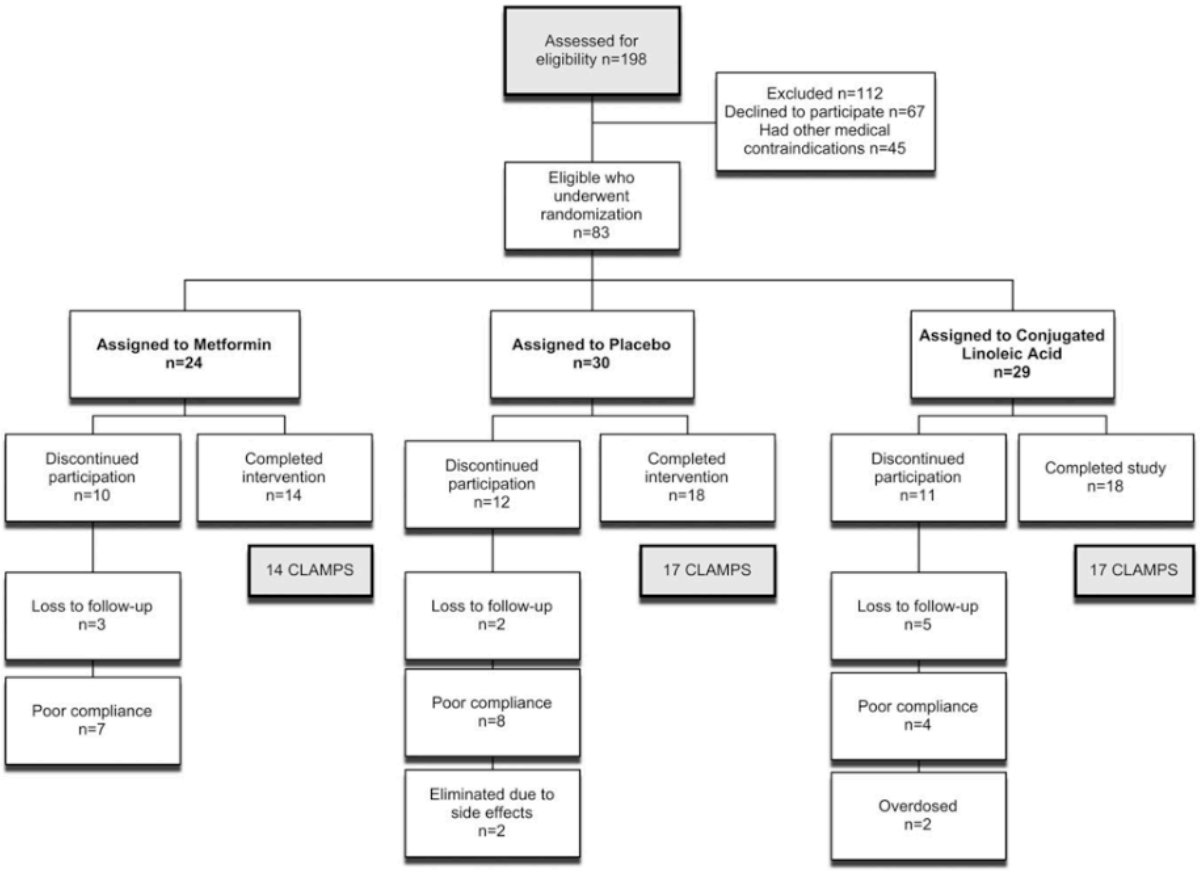

Figure 1. Flowchart representing the number of subjects at study enrollment and study termination. Fourteen clamp studies were conducted in the PLB group, 17 in the MET-treated group, and 17 in the CLA-treated group.

Table 1. Participant Characteristics at Randomization

	MET (n = 14)	Placebo (n = 17)	CLA (n = 17)	*P*
Age, y	11.43 (2.1)	12.59 (2.62)	11.41 (2.71)	0.31
Weight, kg	63.25 (12.6)	70.15 (13.11)	62.11 (18.47)	0.26
Height, cm	148.4 (10)	153.4 (12)	148.6 (13)	0.42
BMI, kg/m^2	28.54 (2.8)	28.79 (2.8)	27.48 (3.7)	0.45
Waist circumference, cm	87.12 (7.6)	89.43 (8.45)	87.17 (11.2)	0.71
Glucose, mg/dL	87 (7)	87 (8.2)	85 (8.1)	0.62
Total cholesterol, mg/dL	167 (27)	167 (28)	162 (23)	0.79
HDL cholesterol, mg/dL	41 (10)	37 (8)	38 (8)	0.42
LDL cholesterol, mg/dL	107 (24)	106 (24)	100 (25)	0.67
Triglycerides, mg/dL	150 (62)	161 (60)	159 (84)	0.89
Physical fitness score (Harvard test)	81 (56)	83 (45)	85 (66)	0.98
Insulin, μU/mL	43.5 (22)	37.1 (17)	42.5 (27)	0.25
HOMA-IR	9.42 (4)	8.07 (4)	9.2 (6)	0.68
QUICKI-IS	0.63 (0.08)	0.64 (0.06)	0.66 (0.15)	0.54

Data are expressed as mean (standard deviation).

Abbreviation: LDL, low-density lipoprotein.

CLA-treated group. However, these effect sizes were not clinically relevant.

We analyzed the changes between initial and final serum triglycerides and high-density lipoprotein (HDL) cholesterol by ANCOVA. For these particular variables, no Tanner or change in BMI modified postintervention levels. Furthermore, HDL cholesterol and triglyceride baseline levels did show an influence over the final levels.

Lipid profile and adverse effects

Patients in the CLA group had a statistically significant increase in serum triglycerides when compared with MET (169.8 ± 69 vs 113.1 ± 27, $P = 0.027$), but not significant when compared with PLB ($P = 0.13$). Moreover, HDL levels were lower in the CLA group when compared with MET (36.8 ± 5.4 vs 44.86 ± 8.7, $P = 0.009$), whereas there were no differences when compared with PLB ($P = 0.26$). The main differences favoring MET treatment over the lipid profile were evident only when compared with CLA.

Nonserious adverse events most commonly reported were abdominal pain, diarrhea, dizziness, headache, nausea, and gastritis. The frequency and severity of symptoms were similar in the three groups (analysis of variance, $P = 0.314$; χ^2 test, $P = 0.28$). Patients exhibiting lack of compliance and/or dropout did not show a difference between groups. Additionally, a Little's missing completely at random analysis ($P > 0.13$) was conducted to ensure that patient elimination was actually random and homogeneous in all of the groups.

Muscle biopsies' analyses

The analyses of IRS1, IRS2, and IRS4 revealed that only IRS2 was modulated in the CLA group, showing a 3.56-fold increase compared with the control group ($P = 0.043$). The rest of the genes did not show statistically

Table 2. Overall Impact of Intervention Regarding Anthropometric, Metabolic, and Insulin Resistance Outcomes in All Patients

	Baseline (n = 48)	4 Months Postintervention (n = 48)	*P*
Weight, kg	65.29 (2.2)	62.51 (2.15)	<0.001
Height, cm	150.2 (1.75)	151.7 (1.66)	<0.001
BMI, kg/m^2	28.25 (0.45)	26.56 (0.51)	<0.001
Waist circumference, cm	87.9 (1.32)	83.8 (1.24)	<0.001
Glucose, mg/dL	87 (1.13)	85 (0.85)	0.28
Total cholesterol, mg/dL	167 (3.75)	161 (4.21)	0.07
HDL cholesterol, mg/dL	39.2 (1.27)	40.4 (1.21)	0.24
LDL cholesterol, mg/dL	105 (3.53)	100 (3.57)	0.056
Triglycerides, mg/dL	154 (9.96)	139 (9.73)	0.22
Insulin, μU/mL	40.9 (3.26)	34.5 (2.95)	0.025
HOMA-IR	8.86 (0.77)	7.27 (0.63)	0.018
QUICKI	0.64 (0.01)	0.68 (0.01)	0.014
Physical fitness score (Harvard test)	83.82 (7.96)	124.0 (5.44)	<0.001

The *P* values were determined by a paired-samples Student *t* test. Data are expressed as mean (SEM).

Abbreviation: LDL, low-density lipoprotein.

Table 3. Characteristics of Main Interest Variables After 4 Months of Intervention

	MET (n = 14)	Placebo (n = 17)	CLA (n = 17)	*P*
Weight, kg	60.85 (3.41)	66.48 (3.19)	59.91 (4.34)	0.39
Height, cm	149.89 (2.45)	154.82 (2.82)	150.27 (3.19)	0.40
BMI, kg/m^2	26.17 (0.99)	27.53 (0.78)	25.92 (0.90)	0.37
Waist circumference, cm	82.31 (1.87)	84.96 (1.77)	83.92 (2.68)	0.70
Prescribed diet compliance, %	92.04 (6.15)	83.78 (6.21)	83.09 (3.56)	0.43
Physical fitness score (Harvard test)	103.82 (16)	132.9 (13.09)	132.79 (14.61)	0.29
Rd value, mg/kg/min	5.57 (0.47)[a,b]	5.05 (0.35)[b]	6.53 (0.61)[a]	0.035
Insulin, μU/mL	40.5 (6.37)	32.3 (4.11)	31.6 (5.05)	0.42
HOMA-IR	8.53 (1.36)	6.83 (0.88)	6.68 (1.08)	0.45
QUICKI-IS	0.65 (0.02)	0.69 (0.02)	0.71 (0.02)	0.38
HDL cholesterol	44.86 (2.33)[a]	40.00 (2.15)[a,b]	36.87± (1.35)[b]	0.031
Triglycerides, mg/dL	113.14 (7.26)[b]	134.12 (20.36)[a,b]	169.80 (15.93)[a]	0.027

Data are expressed as mean (SEM).

[a,b]Homogeneous groups by Fisher's least significant difference contrast.

significant differences. These data support the fact that CLA has a critical effect in the molecular insulin pathway through the upregulation of ISR2. This mechanism might be related to optimal glucose uptake observed in CLA-treated patients.

Discussion

This study supports that CLA improves insulin sensitivity, as measured by EHCT in a group of obese children, and exceeds LIP benefits.

Because the prevalence of metabolic syndrome in our pediatric clinic averages 35% and confers an 11-fold risk of diabetes during early adult life (19), the exploration of conventional and pharmacological strategies focusing on improving the insulin sensitivity level is imperative. Recent studies have revealed that MET has important effects on insulin sensitivity when compared with PLB, and its use in nondiabetic, obese individuals has been massively extended (6, 20, 21). A systematic review conducted by Brufani *et al.* (6) revealed a significant but moderate benefit of MET on weight reduction and fasting insulin sensitivity compared with PLB or lifestyle interventions alone. Nonetheless, when these outcomes were evaluated by frequently sampled intravenous glucose tolerance test (22) or hyperglycemic clamp technique (23), no significant differences were reported. Wiegand *et al.* (5) demonstrated in a randomized PLB-controlled trial a beneficial effect of MET on the insulin sensitivity index in obese, insulin-resistant adolescents; however, no differences in body composition, weight, or BMI were found. We found in our study a significant improvement in all anthropometric parameters, including weight, BMI, waist circumference, and body composition (fat mass and fat-free mass, data not shown); nonetheless, these results were not significantly different among the treatment groups. To our knowledge, no randomized PLB-controlled trial using EHCT had been executed for the evaluation of MET benefits on insulin sensitivity in children. In the present study, we found no differences on Rd value (mg/kg/min) when comparing MET and PLB in the postintervention period. These data could be consistent with the final results of the Diabetes Prevention Program Research Group (24) that showed that diabetes incidence was better reduced in a LIP group compared with PLB. Nonetheless, improvement in terms of BMI, waist circumference, HDL cholesterol, and triglycerides, with considerable effect sizes (72%, 65%, 37%, and 55%, respectively), favored patients treated with MET in our study.

Several studies have proposed beneficial effects of CLA isomers on body composition, inflammation, and insulin sensitivity, promoting differentiation, lipid metabolism regulation, and apoptotic mechanisms in adipocytes (10–12). Interestingly, evidence has suggested that the *trans*-10,*cis*-12 isomer of CLA might induce insulin resistance, whereas the CLA mixture has beneficial effects on body composition and insulin sensitivity. Risérus *et al.* (25) demonstrated that *trans*-10,*cis*-12 isomer–treated subjects presented insulin and glucose increases and decreased HDL and insulin sensitivity measured by 2-hour EHCT compared with PLB or CLA mixture-treated groups. No differences were observed when comparing PLB and CLA mixture-treated individuals. In our study, we showed that the CLA mixture was associated with a clinically relevant effect size (37%) over the Rd value of insulin sensitivity. The confounding variables included in the ANCOVA model showed a decline on group differences. Among these adjustments, Tanner stage was the main variable that modified insulin sensitivity, and despite our small sample size, the effect size of CLA on the Rd value remained.

Despite the fact that several CLA isomers might have deleterious effects on insulin sensitivity and resistance, certain mixtures may neutralize negative effects and even

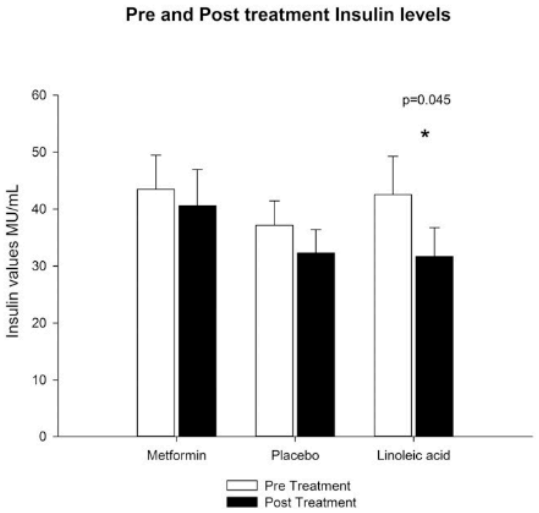

Figure 2. Fasting insulin serum concentrations were significantly lower in the CLA-treated group at study termination, whereas no differences were found in the MET-treated patients or in the PLB group. The error bars show SEM.

induce a synergistic positive response on these parameters, as well as on metabolic and anthropometric values. Some effects of the *trans*-10,*cis*-12 isomer promote a blunted glucose uptake that depends on decreased expression of glucose transporter 4 (GLUT-4) (26). Moreover, decreased incorporation of free fatty acids into the cells may be induced by CLA, a mechanism that could be related to diminished expression of peroxisome proliferator-activated receptor-γ in adipocytes (27). CLA has been proposed as an apoptotic accelerator of adipocytes in mammals that liberates and increases fatty acid oxidation elsewhere in the body (28). Evidence of deleterious effects has mainly been reported in animal models, in which administered doses of CLA are superior to those used in humans (0.2 to 3 g/kg vs 0.015 to 0.1 g/kg, respectively) (29). These effects, if present in humans, could be hyperglycemia and hyperlipidemia, which may predispose an individual to diabetes and nonalcoholic fatty liver disease (30, 31). However, few studies have been published regarding the molecular mechanisms of CLA in skeletal muscle that could explain increased glucose uptake in our treated patients. On this matter, Vaughan *et al.* (32), using a rabdomyosarcoma cell line, have reported that omega-3 fatty acids and CLA activate mitochondrial proliferation and glycolytic activation pathways probably by apoptosis induction and subsequent upregulation of GLUT-4. Furthermore, animal models have shown the beneficial effects of CLA on insulin sensitivity and overexpression of peroxisome proliferator-activated receptor-γ and GLUT-4 in the muscle of supplemented rats (27). In the present study we were able to demonstrate that postintervention IRS2 expression in the skeletal muscle was significantly upregulated in CLA-treated patients. To our knowledge, no studies have been published regarding the effects of CLA or CLA–isomer mixtures on insulin receptor substrate molecules. Xu *et al.* (33) reported that MET upregulates insulin receptor β expression and the downstream IRS2/phosphatidylinositol 3-kinase/Akt signaling transduction in an insulin-resistant rat model of nonalcoholic steatohepatitis and cirrhosis. Our results evidenced a nonsignificant but marginal ($P = 0.055$) IRS2 upregulation in MET-treated children. The insulin-sensitizing effects of MET have been mainly described in liver tissue. Although CLA effects have been mainly focused on adipose tissue modeling, the present study demonstrates that molecular mechanisms, particularly IRS2 upregulation, might mediate insulin-sensitizing effects on skeletal muscle. This phenomenon could explain the increased glucose infusion rate tolerability in our patients treated with CLA throughout the EHCT. Moreover, significant HOMA-IR improvement observed only in CLA-treated patients denotes a significant performance in skeletal muscle that promotes a lower pancreatic insulin secretion.

A recently published meta-analysis demonstrated that the deleterious effects of CLA consumption might be negligible, whereas its benefits, although subtle, seem to be clinically relevant regarding weight and fat mass loss (34). In our study, BMI improvement was significant in all groups, although not significantly different among them. Nonetheless, MET displayed the highest effects over BMI (72%, compared with 43% in the PLB group and 41% in

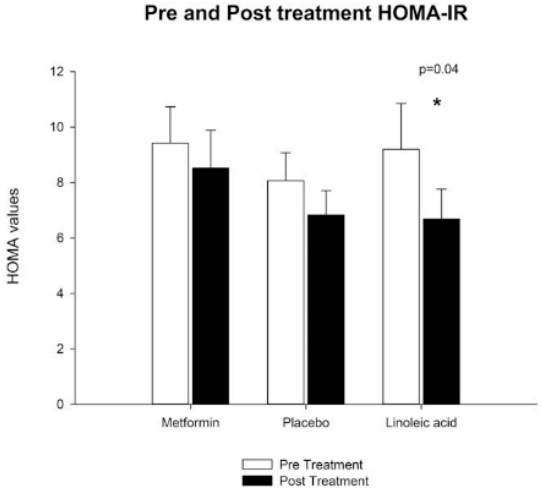

Figure 3. HOMA-IR was significantly lower in the CLA-treated group at study termination compared with initial values. No differences were found in the MET-treated and control groups. The error bars show SEM.

CLA-treated patients) and waist circumference (70%, compared with 60% in PLB and 30% in the CLA group). Total body fat did not improve in any group, but leptin levels significantly decreased in all patients ($P < 0.014$, data not shown).

Racine *et al.* (10) reported a clinical trial in a pediatric population randomly assigned to CLA (3 g/d, *c9,t11-t10-c12*, 50:50) or PLB for 6 months that showed a decrease in total body fat in the CLA group and a significant decrease in HDL cholesterol levels in CLA-treated patients. Our trial demonstrated a significant improvement in HDL cholesterol levels in PLB-treated patients (baseline vs post-intervention, $P = 0.045$). In the CLA group, we noticed a decline in the HDL cholesterol concentration that was not statistically significant when compared with PLB.

One of the limitations of this study was the high rate of participants' withdrawal, as well as the difficulties related to the EHCT, both of which contributed to the final small number of participants, as we did not have enough power for seizing small size effects associated with the treatment.

The strength of this study is supported by its own design. For example, inclusion and, particularly, elimination criteria were strictly applied. Baseline characteristics of participants were similar regarding anthropometric and metabolic condition, particularly those related to surrogated indexes of insulin resistance. Additionally, the main outcome was evaluated by the gold standard EHCT, and the benefits of the overall LIP were evident and similar, regardless of treatment allocation. Although the withdrawal of participants was high in our study, the elimination was random and homogeneous in all groups.

Conclusions

The current study demonstrates the benefits of an LIP and additional effect of CLA over the gold standard EHCT. Lifestyle intervention, independent of any treatment, showed effects on the main outcome variables, specifically weight, height, BMI, waist circumference, surrogate indexes of insulin resistance, and fitness condition, in all of the groups. IRS2 upregulation was evident in CLA-treated patients; this mechanism might be involved in insulin-sensitizing effects on skeletal muscle.

Finally, the incidence of hypertriglyceridemia and hypo-α-lipoproteinemia in CLA-treated patients might be a concern and may be related to the types of CLA isomers used in this study. Further research to evaluate the benefits of different mixtures of CLA isomers may be warranted.

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ORIGINAL ARTICLE

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Analysis of PTPN22, ZFAT and MYO9B polymorphisms in Turner Syndrome and risk of autoimmune disease

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Summary

Turner syndrome (TS) is one of the most common sexual chromosome abnormalities and is clearly associated with an increased risk of autoimmune diseases, particularly thyroid disease and coeliac disease (CD). Single-nucleotide polymorphism analyses have been shown to provide correlative evidence that specific genes are associated with autoimmune disease. Our aim was to study the functional polymorphic variants of PTPN22 and ZFAT in relation to thyroid disease and those of MYO9B in relation to CD. A cross-sectional comparative analysis was performed on Mexican mestizo patients with TS and age-matched healthy females. Our data showed that PTPN22 C1858T (considered a risk variant) is not associated with TS (X^2 = 3.50, p = .61, and OR = 0.33 [95% CI = 0.10–1.10]). Also, ZFAT was not associated with TS (X^2 = 1.2, p = .28, and OR = 1.22 [95% CI = 0.84–1.79]). However, for the first time, rs2305767 MYO9B was revealed to have a strong association with TS (X^2 = 58.6, p = .0001, and OR = 10.44 [95% C = 5.51–19.80]), supporting a high level of predisposition to CD among TS patients. This report addresses additional data regarding the polymorphic variants associated with autoimmune disease, one of the most common complications in TS.

KEYWORDS
autoimmune disease, MYO9B, PTPN22, turner syndrome, ZFAT

1 | INTRODUCTION

Turner syndrome (TS) is one of the most common sexual chromosome abnormalities, affecting 1/2500 live female births (Pinsker, 2012). It is associated with a threefold increase in mortality and a 13-year decrease in life expectency (Stochholm, Juul, Juel, Naeraa, & Gravholt, 2006). TS is characterized by total or partial monosomy of the second sex chromosome, short stature, skeletal abnormalities and gonadal dysgenesis, accompanied by a lack of estrogens throughout life. The most common systemic complications are heart and renal defects (Carvalho et al., 2010) and osteoporosis (Gravholt et al., 2003) and, in adulthood, metabolic syndromes (Davenport, 2010) and autoimmune diseases, including hypothyroidism (Fukuda et al., 2009). Haploinsufficiency of genes on the X chromosome may be responsible for a lack of self-protein exposure in the thymus and the escape of auto-reactive T-cells, thus predisposing patients to autoimmunity (Bianchi, Ileo, Gershwin, & Invernizzi, 2012). Among the autoimmune disorders associated with TS, Hashimoto's thyroiditis has been estimated to affect around 50% of patients (Bakalov et al., 2012; Larizza, Calcaterra, & Martinetti, 2009). Other associated autoimmune disorders include inflammatory bowel diseases, such as coeliac disease (CD) (Dias Mdo et al., 2010; McCarthy & Bondy, 2008), ulcerative colitis and Crohn's disease. (Bakalov et al., 2012; Davenport, 2010). The risk of CD in TS patients during the first 5 years of life is approximately twofold higher than normal and increases after 10 years to more than fivefold higher than normal. The relevant data are the medical follow-ups of these patients. During childhood, there is close medical

supervision; however, in adulthood, close surveillance by a multidisciplinary group often decreases, despite a high rate of complications and the fact that the quality of life for adult TS patients is improved due to multidisciplinary management and medical follow-up; most TS patients require long-term monitoring and early treatment to avoid complications (Donaldson, 2006; Elsheikh, Dunger, Conway, & Wass, 2002). In order to improve the clinical approach and attempt to identify those patients with a high risk of autoimmune disease, genetic medical groups who attend to these patients have conducted formal studies analysing the polymorphic variants of genes associated with TS. In 2015, a systematic review of the formal studies of polymorphisms associated with the aetiology and clinical manifestations of TS found 17 studies in PubMed suggesting that identifying such polymorphisms could be a useful strategy in identifying high-risk groups. However, considering the small number of published studies and contradictory findings related to the varying ethnicities present in the analysed samples, studies carried out in different populations are needed to clarify the role of these single-nucleotide polymorphisms (SNPs) in the aetiology and clinical manifestations of this chromosomal disorder (Trovó de Marqui, 2015). Therefore, the aim of this study was to determine the allelic frequency of three functional polymorphisms associated with autoimmunity in 109 Mexican TS patients. PTPN22 C1858T, a well-known variant associated with autoimmune disease in women, was previously associated with thyroid disease in Brazilian TS patients (Bianco et al., 2010). In an attempt to improve screening for thyroiditis, this study analysed, in addition to PTPN22 C1857T, ZFAT EX9b-SNP10 polymorphism, which is associated with susceptibility to autoimmune thyroid diseases, mainly Graves and Hashimoto thyroiditis. This study also explores CD, the second most frequent autoimmune complication of TS. For this reason, we also analysed the MYO9B rs2305767 variant, which is strongly associated with CD and inflammatory bowel disease, in the Latin American population.

2 | MATERIALS AND METHODS

2.1 | Study design and sample size calculation

A comparative cross-sectional analysis of TS patients vs. healthy females was performed. To make an accurate sample calculation with the necessary power to associate the variant in question with TS, an independent proportion comparison formula was used. Using information in Bianco et al. (2010), data with an alpha of 0.05 and 90% power were used. The sample size calculation yielded 47 TS patients. This sample size was increased to 109 TS patients (73 paediatric patients and 36 adult patients) to obtain a consistent data. The control group was composed of 109 healthy females with no medical history of autoimmune disease for at least two generations. The TS cases were age-matched with the healthy controls.

2.2 | Cytogenetic and genetic background

The diagnosis of chromosomal abnormalities was performed via GTG cytogenetic analysis (Table 1). The TS patients did not have to exhibit autoimmunity to be included. To control for ethnicity, all the subjects included in the study had a Mexican-mestizo genetic background.

TABLE 1 Cytogenetic analysis of TS patients

Karyotype	Number of patients
45,X	79
45,X/46,XX	6
45,X/46,XY	10
46, X iso (Xq)	14
Total	109

2.3 | Molecular analysis

Peripheral blood was collected from each patient and each control in an EDTA-containing tube. Genomic DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen, Maryland, USA). Total DNA was determined by measuring OD at 260/280 nm using a Nano-Drop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The integrity of the DNA was determined using 1% agarose gel electrophoresis. All the samples included had the quality parameters necessary for the genotyping. The genotyping of the PTPN22 C1858T polymorphism, (rs2476601) ZFAT Ex9b-SNP10 (rs16905194) and MYO9B (rs2305767) was performed using a TaqMan SNP genotyping real-time PCR assay from Applied Biosystems (Foster City, CA, USA). The assays were performed with TaqMan Universal Master Mix (Applied Biosystems), using 10–20 ng of DNA per reaction. The PCR conditions were those recommended by the manufacturer: initial denaturation at 95°C (15 min), followed by 40 denaturation cycles at 95°C (15 s), and a final annealing/extension cycle at 60°C (1 min).

The probe sequences are as follows:

PTPN22:(VIC/FAM)ACCACAATAAATGATTCAGGTGTCC(A/G)
TACAGGAAGTGGAGG GGGGATTTCA
ZFAT:(VIC/FAM)ACAGAAATTTTATTATCGTAGCATA(A/T)
AGCACAACTGCCACTTAACAGC ACA
MYO9B:(VIC/FAM)GTCAGTTCCTCCATAGCAAGCCCCG(C/T)
TGGATGCACGTCCCACCCTGTAGAT

2.4 | Statistical analysis

A chi-square test was used to compare the allele and genotype frequencies between the groups, estimate the Hardy–Weinberg equilibrium, and assess potential associations. Statistical tests were carried out using SPSS for Windows 11.0 (SPSS, Inc., Chicago, IL, USA). All *p*-values were two-tailed and included 95% confidence intervals (CIs), and *p*-values <.05 were considered statistically significant.

3 | RESULTS

All the analysed polymorphisms in our population were in Hardy–Weinberg equilibrium. The PTPN22 C1858T polymorphism previously associated with a 2.2 OR in a Brazilian TS population showed clear

TABLE 2 Genotype and allelic frequencies of the rs 2476601 *PTPN22 C1858T* polymorphism

	PTPN22 genotypes		Alleles					
	CC n(%)	CT n (%)	TT n (%)	C n (%)	T n (%)	*p*	OR [CI 95%]	*N*
TS	104 (95.41)	4 (3.66)	1 (0.91)	212 (97.24)	6 (2.75)	.61	0.33 [0.10–1.10]	109
Controls	97 (88.9)	11 (10)	1 (0.9)	205 (94.03)	13 (0.59)			109

TS, Turner Syndrome; OR, Odds Ratio.
$p < .05$ was considered statically significant.

differences in our Hispanic population. The risk allele CT was present in 3.66% (4/109) of the TS population, whereas the non-deleterious variants were present in 96.31% of the TS population (105/109). In the control group, the allelic distribution showed that the risk allele CT was present in 10% of that group (11/109), and the TT and CC alleles were observed in 89.8% of that group (98/109) (Table 2). The statically analysis showed no significance or association with the syndrome (Table 2). The ZFAT Ex9b-SNP10 rs16905194 polymorphism, which is associated with thyroid autoimmunity, was also not associated with TS; the genotype and allelic frequencies are shown in Table 3. The statistical analysis showed an $X^2 = 1.2$, $p = .28$, and an OR = 1.22 [95% CI = 0.84–1.79]. The MYO9B rs2305767 variant was included to explore whether it was associated with CD risk in the TS group, which revealed that the frequency of the CT risk genotype in the TS group was 82.5% (90/109). The non-deleterious genotypes, CC and TT, were observed in 17.42% of the TS group (19/109) (Table 4). In the healthy control group, the risk variant was seen in 31.19% (34/109) of individuals, while the TT and CC genotypes were seen in 68.8% (75/109) of individuals. Statistical analysis showed $X^2 = 58.6$ and $p = .0001$, with an OR = 10.44 [95% CI = 0.84–1.79] (Table 4). These data demonstrate a strong correlation between the risk allele and the TS group and no such correlated in the control healthy group.

4 | DISCUSSION

TS patients have a high risk of developing autoimmune diseases as common systemic complications. This research paper presents the results of a cross-sectional study of 109 Mexican TS patients examining two well-known polymorphisms associated with thyroid disease, PTPN22 and ZFAT, and a third variant in the MYO9B gene associated with CD. It is well-known that there is an increased incidence of autoimmune disease associated with TS (Dias Mdo et al., 2010; McCarthy & Bondy, 2008). In particular, an increased frequency of thyroid abnormalities and bowel inflammatory disease, such as Hashimoto's thyroiditis, CD, Crohn's disease and ulcerative colitis, has been observed (Durusu, Gurlek, Simsek, Balaban, & Tatar, 2005; El-Mansoury et al., 2005; Ivarsson et al., 1999; Mortensen et al., 2009). Jørgensen et al. (2010) found an overall increased risk of autoimmune diseases in a cohort of 798 women with TS independent of the karyotype giving rise the syndrome. All the cytogenic formulas were associated with a two- or threefold increase in the risk of developing an autoimmune disease, and this risk increased with the age. This is important due to the observed decrease in medical surveillance during adulthood, despite a high rate of complications. Treatment and follow-up have improved the quality of life of adult patients in recent years, and

TABLE 3 Genotype and allelic frequencies of the rs16905194 *ZFAT* polymorphism

	ZFAT genotypes			Alleles				
	AA n(%)	AT n(%)	TT n(%)	T n(%)	A n(%)	*p*	OR [95% CI]	*N*
TS	26 (23.85)	64 (58.71)	19 (17.43)	102 (46.78)	116 (53.21)	.28	1.22 [0.84–1.79]	109
Controls	39 (35.77)	49 (44.95)	21 (19.26)	91 (41.74)	127 (58.25)			109

TS, Turner Syndrome; OR, Odds Ratio.
$p < .05$ was considered statically significant.

TABLE 4 Genotype and allelic frequency of the *rs* 2305767 *MYO9B* polymorphism

	MYO9B Genotypes			Alleles				
	CC n(%)	CT n(%)	TT n(%)	C n(%)	T n(%)	*p*	OR [CI 95%]	*N*
TS	2 (1.83)	90 (82.56)	17 (15.59)	94 (43.11)	124 (56.88)	.0001	10.44 [5.51–19.80]	109
Controls	2 (1.83)	34 (31.19)	73 (66.97)	38 (17.43)	180 (82.56)			109

TS, Turner Syndrome; OR, Odds Ratio.
$p < .05$ was considered statically significant.

most TS patients require long-term monitoring and early treatment to avoid complications (Donaldson, 2006). Our experience in a general reference hospital showed that only 5% of TS patients received long-term continuous and multidisciplinary attention; this number is similar to those seen in previous reports in the literature (Elsheikh et al., 2002). On the other hand, SNP analyses have shown correlative evidence for the involvement of specific genes in human disease. Some of these variants are already useful in clinical practice, allowing the identification of high-risk populations with the intention to improve preventive medicine. TS is no exception. However, in 2015, a systematic review of formal studies of polymorphisms associated with the aetiology and clinical manifestations of TS concluded that the evidence is insufficient and more studies are needed to highlight the clinical relevance of this association and the influence of ethnicity (Trovó de Marqui, 2015). Bianco et al. reported that the PTPN22 C1858T variant could be an important genetic factor in autoimmune disease risk in the Brazilian TS population. This gene encodes a lymphoid-specific phosphatase (LYP), an important down-regulator for T-cell activation that is associated with the development of autoimmunity processes (Begovich et al., 2004; Bottini, Vang, Cuccam, & Mustelin, 2006; Kyogoku et al., 2004). This genetic variant was analysed in 109 TS patients and compared with 109 healthy, age-matched controls. In our study group, PTPN22 was not related with the syndrome (p = .61, OR 0.33), confirming that this polymorphism has a different effect in different populations and highlighting the importance of genetic background (Liao, Chen, Zhao, & Xie, 2015; Trovó de Marqui, 2015). Bearing in mind that polymorphisms act in an additive manner, the ZFAT Ex9b-SNP10 variant was included in an attempt to explore whether other polymorphic variants can be associated with thyroid autoimmunity risk in TS. This allelic variant had very consistent results in the literature associated with thyroid autoimmunity in females, and no previous studies had been performed regarding this group of patients. ZFAT is expressed on B and T lymphocytes and has been shown to be a critical transcriptional regulator (Koyanagi et al., 2008; Shirasawa et al., 2004) and anti-apoptotic molecule in lymphocytes (Fujimoto et al., 2009). The Ex9b-SNP10 (rs16905194) polymorphism in the ZFAT gene has been found to be associated with susceptibility to autoimmune diseases. We believed that the ZFAT variant was a very good candidate to explore in our population. Nevertheless, the negative association results (OR = 1.22, p = .28) indicate that in our patients, it was not related with autoimmunity disease risk among those with TS. Both polymorphisms included were previously associated with thyroid autoimmunity in a female population, but were not useful in identifying a group at risk of thyroiditis in Mexican TS patients. It is clear that it could be interesting to analyse other variants associated with thyroid disease, such as CTLA4, CD40 or FCRL3 (Inoue et al., 2012). Finally, we considered taking CD into account to explore its relationship with the syndrome. Is it well-known that the prevalence of CD diagnoses is greatly underestimated in TS cases. In a recent analysis of the Swedish population, a case–control study found that the risk of TS in the first 5 years or life is 2.16 times higher and increases to an OR of 5.5 after 10 years. These data confirm that CD should always be considered in the clinical evaluation of TS patients throughout their lives, though this does not necessarily happen. The analysis of the MYO9B myosin IXB (*MYO9B*) gene, which is associated with susceptibility to CD (Bähler, Kehrer, Gordon, Stoffler, & Olsen, 1997), would be an interesting approach to identifying an at-risk group among TS patients. *MYO9B* encodes a Ras homologous (Rho) family guanosine-tri-phosphatase (GTPase)-activating protein that is involved in epithelial cell cytoskeletal organization and influences tight junction assembly (Matter & Balda, 2003; Post et al., 2002). Human MYO9B is expressed in intestinal epithelial cells, and increased *MYO9B* gene expression is correlated with increased intestinal permeability in vivo (Sapone et al., 2006). Many SNPs have been identified in the *MYO9B* gene. Four of these polymorphisms are the most frequently analysed: rs1545620 A>C (exon 20), rs1457092 C>A (intron 20), rs2305767 A>G (intron 14) and rs2305764 A>G (intron 28). The behaviours of each of the SNPs have an ethnic background. Several association studies have evaluated the relationship between these polymorphisms and inflammatory bowel diseases, including CD. We analysed rs2305767 in a Latin American population. Interestingly, the data showed a strong association with the risk variant in the TS population as compared with the controls (OR 10.44, p = .0001). These data and the literature support the fact that this variant could be useful in identifying a high-risk group within the TS population. In a recent paper in which a meta-analysis was performed to clarify the association between genetic variants and the risk of developing CD, the authors concluded that the rs2305767 variant is highly associated with the risk of CD in the Latin American population, whereas the rs1545620 variant is associated with the risk of CD in the European population (Liao et al., 2015). These results confirm and support our observation. To our knowledge, this is the first report to find that a polymorphism associated with CD in Latin Americans is also associated with TS patients. As part of the follow-up, our clinic is following a cohort of patient with the CT genotype, which is considered a high-risk allele in TS patients. A cohort of 90 TS patients is being studying. Most of them are paediatric cases, and until now, none of them have developed symptoms. This group of patients is followed to determine which is the CD behaviour. In conclusion, we have not identified a genetic variant associated with thyroid disease that can be used in the clinic. However, a strong association was observed for MYO9B, a gene closely related with CD, which could be used to improve the detection of the disease in TS cases. This report addresses additional data regarding the polymorphic variants associated with autoimmune diseases, one of the most common complications in TS.

DECLARATION OF CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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