UNIVERSIDAD AUTÓNOMA DE GUERRERO

UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS
UNIDAD DE INVESTIGACIÓN ESPECIALIZADA EN MICROBIOLOGÍA

DOCTORADO EN CIENCIAS BIOMÉDICAS

FACTOR INHIBIDOR DE LA MIGRACIÓN DE MACRÓFAGOS Y SUBTIPOS DE MONOCITOS-MACRÓFAGOS Y DE LINFOCITOS T COOPERADORES COMO MARCADORES DE INFLAMACIÓN EN OBESIDAD

T E S I S

QUE PARA OBTENER EL GRADO DE

DOCTORADO EN CIENCIAS BIOMÉDICAS

P R E S E N T A:

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ACTA DE APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 02 días del mes de diciembre del dos mil catorce, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado del Doctorado en Ciencias Biomédicas, para examinar la tesis titulada **"Factor inhibidor de la migración de macrófagos y subtipos de monocitos-macrófagos y de linfocitos T cooperadores como marcadores de inflamación en obesidad"**, presentada por la alumna Ines Matia Garcia, para obtener el Grado de Doctora en Ciencias Biomédicas. Después del análisis correspondiente, los miembros del comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

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Este trabajo de investigación se realizó en el Laboratorio de Investigación en Obesidad y Diabetes (LIOD) de la Unidad Académica de Ciencias Químico Biológicas, de la Universidad Autónoma de Guerrero.

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ÍNDICE

RESUMEN

Introducción. La inflamación sistémica crónica que se presenta en individuos obesos, se caracteriza por el incremento en las células del sistema inmune innato y adaptativo, así como el aumento en los niveles sanguíneos de citocinas y quimiocinas. Actualmente, se considera que esta inflamación crónica puede ser uno de los mecanismos que contribuyen al desarrollo de la resistencia a la insulina (RI), diabetes mellitus tipo 2 (DMT2), aterosclerosis y enfermedades cardiovasculares. **Objetivo.** Analizar el estado inflamatorio en jóvenes con y sin obesidad, determinando los subtipos de linfocitos Th, de monocitos-macrófagos, niveles de citocinas, así como la relación de variantes en el gen *MIF* con su expresión de mRNA y niveles circulantes de la proteína. **Materiales y métodos.** Se incluyeron 250 jóvenes: 150 con peso normal y 100 con obesidad. La identificación de los subtipos de linfocitos Th (Th1 y Th2) y subtipos de monocitos-macrófagos se realizó por citometría de flujo. La genotipificación de los polimorfismos -794 $CATT_{5-8}$ y -173 G>C en el gen *MIF* se realizó por PCR-RFLP; la expresión de su mRNA se determinó por PCR en tiempo real y los niveles séricos de MIF se midieron utilizando un kit de ELISA. Las concentraciones de 10 citocinas se midieron con un sistema multiplex basado en perlas y los niveles séricos de hsCRP se determinaron por turbidimetría. **Resultados.** Los jóvenes con obesidad presentaron un mayor número de células T $CD3^+CD4^+$, así como de células Th1 y una disminución de células Th2 en comparación con los de peso normal, además tuvieron un aumento de las subpoblaciones de monocitos-macrófagos $CD68^+CD14^-$, $CD16^+CD14^-$ y $CD16^{low}CD14^-$. Tanto las células Th1 como los monocitos-macrófagos se correlacionaron positivamente con medidas de adiposidad. La expresión del mRNA de *MIF* se encontró incrementada en obesos con respecto a los de peso normal (p= 0.38), así como en los portadores de los genotipos -794 $CATT_{66}$ (p= 0.55) y -173CC (p= 0.45) y el haplotipo 6G (p= 0.56) de los polimorfismos -794 $CATT_{5-8}$ y -173 G>C en el gen *MIF*, aunque esta diferencia no fue significativa. Además, se observó una correlación positiva entre las medidas de adiposidad y los niveles sanguíneos de IL-6 y hsCRP, pero una correlación negativa con IL-10. **Conclusión.** Los jóvenes con obesidad presentan mayor número de células Th1 y monocitos-macrófagos y una

disminución de las Th2, un aumento en los niveles séricos de IL-6 y hsCRP, así como un leve incremento en la expresión del mRNA de *MIF* en comparación con los de peso normal, lo que sugiere que la desregulación metabólica causada por la obesidad puede ser modulada por las células del sistema inmune innato y adaptativo y citocinas proinflamatorias que en conjunto contribuyen a la fisiopatología de la obesidad.

Palabras claves: Obesidad, linfocitos T, monocitos-macrófagos, factor inhibidor de la migración de macrófagos, polimorfismos, inflamación.

INTRODUCCIÓN

La obesidad es una enfermedad crónica y multifactorial, cuya prevalencia va en aumento a nivel mundial. Este incremento se puede atribuir a la disponibilidad y al consumo de alimentos con alto contenido de grasa, en combinación con un estilo de vida sedentario (Tateya *et al.,* 2013). En diversos estudios se ha identificado que la obesidad y principalmente el aumento de adiposidad en la región abdominal, se asocia con inflamación de grado bajo, resistencia a la insulina (RI), homeostasis alterada de la glucosa y con sus comorbilidades tales como la diabetes mellitus tipo 2 (DMT2), la hipertensión, las dislipidemias y las enfermedades cardiovasculares (Hotamisligil *et al.,* 2006; Despres *et al.,* 2008; Nishimura *et al.,* 2009; Kleemann *et al.,* 2010).

Actualmente, se considera al tejido adiposo (TA) como un órgano endocrino activo que secreta mediadores importantes de la inflamación, tales como interleucina 6 (IL-6), factor de necrosis tumoral alfa (TNF-α), resistina, interleucina 8 (IL-8), proteína quimioatrayente de monocitos 1 (MCP-1), interleucina 1-β (IL-1β) y el factor inhibidor de la migración de macrófagos (MIF). La red de citocinas favorece la producción de reactantes de fase aguda como la proteína C reactiva (CRP), la haptoglobina y el fibrinógeno, que en conjunto contribuyen a un estado de inflamación sistémica crónica de grado bajo característico de la obesidad; además de que algunas de estas adipocinas se encuentran implicadas en el desarrollo de la RI y síndrome metabólico (Gustafson *et al*., 2007; Sakaue *et al.,* 1999; Nishimura *et al.,* 2009; Lumeng *et al.,* 2011). Varios estudios han mostrado que niveles séricos de IL-6, MCP-1, IL-8, MIF y proteína C reactiva (CRP) se correlacionan positivamente con peso, índice de masa corporal (IMC), circunferencia de cintura y otros factores tradicionales de riesgo cardiovascular (Dandona *et al.,* 2004; Khaodhiar *et al.,* 2004; Kim *et al.,* 2006). Además, varios estudios muestran que individuos con glucosa alterada o con DMT2 presentan niveles elevados de IL-6, IL-8, CRP y MIF. También se conoce que la IL-6 y TNF-α interfieren en la vía de señalización de la insulina y reducen la respuesta de los hepatocitos y músculo a la acción de la insulina (Herder *et al.,* 2005, 2006; Weigelt *et al.,* 2009; Fain *et al*; 2006). Estudios previos han mostrado que MIF por su actividad proinflamatoria, juega un papel importante en la patogénesis de DMT2

(Sánchez-Zamora *et al.,* 2010; Hender *et al.,* 2008). En estudios realizados *in vitro* en células *β* pancreáticas, se observó que MIF se produce en estas células y se localiza con la insulina en los gránulos secretores. La producción del MIF fue dependiente de la glucosa, regulando la liberación de la insulina de manera autocrina. Además, se observó que mediante la inmunoneutralización del MIF se disminuye la secreción de insulina inducida por la glucosa, en contraste a la exposición de MIF recombinante exógeno, que potenció la liberación de la insulina de los islotes pancreáticos (Waeber *et al.,* 1997). Por otra parte, se ha demostrado *in vitro*, que la glucosa e insulina regulan la expresión de MIF en adipocitos 3T3-L1. En otro estudio, encontraron que los ratones MIF$^{-/-}$ producen menos IL-6, TNF-α e IL-1β en comparación con los MIF $^{+/+}$, lo que sugiere que MIF puede contribuir a la patogénesis de DMT2 al inducir la producción de citocinas proinflamatorias y/o al modular la función de los adipocitos (Sakaue *et al.,* 1999; Plaisanse *et al.,* 2002; 2010; Sánchez-Zamora *et al.,* 2010).

La inflamación de bajo grado en la obesidad y la DMT2 se considera el producto de un sistema inmune innato activado, lo que desencadena la infiltración de células inflamatorias, tales como macrófagos, mastocitos, neutrófilos, células dendríticas y linfocitos, resultando en alteraciones cualitativas y cuantitativas de la fracción del estroma vascular del TA blanco (Weisberg *et al.,* 2003; Xu *et al.,* 2003; Chmelar *et al.,* 2013). En el 2003, dos estudios independientes demostraron que la expansión del TA en ratones obesos se acompaña por una infiltración progresiva de monocitos en este tejido, lo cual se puede atribuir a que los adipocitos producen CSF-1 el regulador primario de la diferenciación y supervivencia de macrófagos así como de citocinas proinflamatorias, tales como MIF, IL-6, TNF-α y MCP-1, por lo tanto a mayor adiposidad, el TA puede secretar más quimiocinas y CSF-1, llevando al reclutamiento de más monocitos que se diferencian a macrófagos en el TA (Weisberg *et al.,* 2003; Xu *et al.,* 2003;). Se conoce que la diferenciación de monocitos a macrófagos es mediada por el factor estimulador de colonias de macrófagos (M-CSF), y su polarización a dos subtipos: macrófagos M1 y M2 puede estar determinada por células T-helper. En la polarización hacia macrófagos M1 o activados clásicamente, participan lipopolisacárido (LPS), y citocinas liberadas por células T-helper 1 (Th1) incluyendo IFN-γ, interleucina 2 (IL-2) y TNF-α, que llevan a la producción de altos

niveles de IL-12, IL-23, IL-6 y TNF-α. El subtipo de macrófagos M2 o activados alternativamente, en contraste a los M1, son estimulados por las citocinas de tipo Th2, interleucina 4 (IL-4) e interleucina-13 (IL-13), y producen grandes cantidades de IL-10, estos macrófagos expresan arginasa 1, el receptor de manosa (CD206) y CD301 (Gordon *et al.,* 2003; Martinez *et al.,* 2006; Chawla *et al.,* 2011).

En la obesidad el balance entre los macrófagos M1 y M2 está alterado y se considera que los macrófagos son más abundantes en el tejido adiposo visceral que en el subcutáneo. Se ha reportado que los macrófagos que se acumulan en el tejido adiposo de ratones obesos, expresan principalmente genes asociados con un fenotipo de macrófagos M1 o activados clásicamente, mientras que en ratones delgados expresan genes asociados con un fenotipo de macrófagos M2 o activados alternativamente, que secretan citocinas anti inflamatorias como IL-4, IL-10 e IL-13. Los macrófagos M1 secretan mayor cantidad de citocinas proinflamatorias como TNF-α, IL-1β, IL-6, MCP-1 y MIF, este último activa y recluta más macrófagos al tejido adiposo y se sugiere que los lleva hacia el fenotipo M1. En el 2007, en un estudio en ratones, se propuso un modelo de "cambio de fenotipo" el cual sugiere que en un ambiente obesogénico, los macrófagos asociados al tejido adiposo son polarizados de un estado de activación M2 a M1. En el 2009, Wisnewsky *et al.,* realizaron un estudio en adultos obesos y mostraron que la pérdida de peso altera significativamente la expresión de marcadores de superficie de los macrófagos M1 y M2, promoviendo un menor perfil inflamatorio (Lumeng *et al.,* 2008; Aron-Wisnewsky *et al.,* 2009).

En un estudio en individuos obesos con DMT2, reportaron que la restricción calórica con una modesta pérdida de peso disminuye el número de monocitos, además observaron un gran número de macrófagos y células precursoras CD34+ en tejido adiposo subcutáneo comparado con tejido adiposo visceral (macrófagos 6.7±1.8 *vs* 2.9±1.0%, p=0.02; células precursoras 7.0±2.7 vs 1.8±0.3%, p=0.04), en contraste a lo reportado en otro estudio realizado por Harman-Boehm, en el 2007 donde encontraron una mayor infiltración de macrófagos en el tejido adiposo omental comparado con el subcutáneo (Viardot *et al.,* 2010; Harman-Boehm *et al.,* 2007). En

otro estudio en individuos obesos, después de una dieta hipocalórica o cirugía gástrica, la pérdida de peso llevó a una disminución de los subtipos de monocitos $CD14^{dim}CD16^{+}$ y $CD14^{+}CD16^{+}$ (Poitou *et al.,* 2011).

La contribución del sistema inmune adaptativo en la obesidad, en particular las células T no se ha estudiado lo suficiente; sin embargo, la evidencia experimental de que los linfocitos son importantes en la inflamación del TA blanco en obesos está emergiendo. La alteración en el equilibrio entre diferentes poblaciones de células T dentro del tejido adiposo visceral, parece ser un evento temprano en la obesidad. Definidas por su perfil de citocinas secretadas, las células T helper $CD4^{+}$ pueden diferenciarse en un tipo proinflamatorio 1 (Th1) que producen IFN-γ, IL-2, e IL-12, o un tipo 2 (Th2) que reprime a las Th1 por la producción de IL-4, IL-5, IL-10 e IL-13. La vía Th1 se asocia con la inducción de la inmunidad celular, es decir la activación de linfocitos $TCD8^{+}$, células NK y macrófagos. Las citocinas Th2 apoyan la respuesta humoral relacionada a la función de los linfocitos B. Un balance entre estas dos vías se considera esencial en la regulación inmune (Rogge, 2002; Romagnani, 1996; Zhu *et al.,* 2008).

En 2009 tres estudios independientes demostraron el papel clave de las células T que infiltran el TA blanco para el desarrollo de RI y el fenotipo inflamatorio asociado en modelos genéticos obesos (Feuerer *et al.,* 2009; Nishimura *et al.,* 2009; Winer *et al.,* 2009). En estudios previos en humanos y ratones obesos, se ha observado que la expansión del TA visceral lleva a una mayor infiltración de células T a este tejido (Wu *et al.,* 2007; Rocha *et al.,* 2008*;* Duffaut *et al.,* 2009). Estudios recientes indican que la obesidad no sólo se caracteriza por un aumento de la activación de células inmunes circulantes del sistema inmune innato sino también del adaptativo. Viardot *et al.,* realizaron un estudio de intervención en sujetos obesos mórbidos con DMT2 y observaron qué efecto tendría la pérdida de peso sobre células inmunes, y reportaron que hubo una reducción significativa en el número de células Th1 (5.3±1.6 a 0.8±0.2%, p=0.03), en la proporción de Th1 a Th2 (2.5±0.6 a 0.6±0.1, p=0.01), en la expresión de marcadores de activación de superficie en linfocitos T (CD69 y CD25), y poco efecto sobre el número de células Th2, lo que sugiere que la pérdida de peso induce cambios notables en la activación y fenotipo de las células inmunes,

resultando en un mayor equilibrio de células antiinflamatorias (Viardot *et al.,* 2010). El aumento en la secreción de citocinas Th1 puede ser considerado como un mecanismo que contribuye a la inflamación en la obesidad (Pacifico *et al.,* 2006). Los hallazgos descritos pueden sugerir que la inflamación sistémica en la obesidad se caracteriza por un sistema inmune adaptativo sobre-activado.

Se conoce que MIF fue una de las primeras citocinas proinflamatorias que se describió (en 1966), como un factor soluble expresado por linfocitos T en respuesta a la hipersensibilidad retardada y ejerce un efecto inhibitorio sobre la migración de los macrófagos *in vitro* (David, Bloom, 1966). Se han realizado estudios que demuestran que MIF es un importante regulador de la respuesta inmune innata (Calandra *et al.,* 2003). MIF se produce por diferentes tipos de células y tejidos como, células T, macrófagos, monocitos, glándula pituitaria, fibroblastos, células endoteliales y adipocitos (Donn, 2004; Surk *et al.,* 2005; Grieb *et al.,* 2010). MIF estimula la expresión y secreción de citocinas proinflamatorias como TNF-α, IFN-ɣ, IL-1β, IL-6, IL-2 e IL-8 y también contra regula el efecto antiinflamatorio de los glucocorticoides (Sánchez-Zamora *et al.,* 2010; Calandra *et al.,* 1995; Flaster *et al.*, 2007). Se ha determinado que los niveles del MIF se encuentran incrementados en la artritis reumatoide (AR), sepsis severa, obesidad y DMT2, enfermedades que cursan con inflamación persistente de diferente grado (Baugh *et al.,* 2002; Llamas-Covarrubias, *et al.,* 2012; Lehmann *et al.,* 2009; Dandona *et al.,* 2004; Vozarova *et al.,* 2002). Por lo que es importante realizar estudios para tratar de dilucidar el papel del MIF en su desarrollo.

La estructura de MIF es única, es una proteína formada por 115 aminoácidos, un peso de 12.5 kDa. La estructura cristalina de MIF, muestra que se constituye en un trímero de 3 subunidades idénticas. Cada monómero de MIF consiste de dos hélices α anti paralelas (α1 y α2) y seis cadenas β (β1-β6). Cuatro de las seis cadenas β (β1, β2, β4, β5) forman una hoja plegada β, y las dos cadenas β restantes interactúan con las hojas β de las subunidades adyacentes, para formar la interface

entre monómeros (Sun *et al.,* 1996; Orita *et al.*, 2001; Donn*,* 2004; Tillmann *et al.,* 2013).

Por otro lado, Los macrófagos son una fuente primaria de MIF *in vitro* e *in vivo* (Calandra *et al.* 1994). MIF se secreta en respuesta a estímulos inflamatorios como LPS, TNF-α e IFN-ɣ (Calandra *et al.,* 2003, 2004; Baugh *et al.,* 2002). Roger *et al.,* demostraron que los macrófagos MIF -/- tuvieron una baja respuesta al LPS, debido a la reducción en la actividad de NF-κB y la producción de TNF-α (Roger *et al.,* 2001). También se ha determinado que MIF endógeno, regula la inmunidad innata a través de la sobre expresión del receptor tipo toll 4 (TLR4), el receptor de IL-1 (IL-1R) y el receptor para TNF (TNFR) (Roger *et al.,* 2001; Toh *et al.,* 2006). Además, MIF disminuye la apoptosis dependiente de p53 lo que conlleva a un incremento de la vida media de los macrófagos activados, y por lo tanto a una amplificación de la respuesta inflamatoria (Mitchell *et al.,* 2002). Debido a su funcionalidad y prominente papel en la biología de los macrófagos, así como su propiedad quimioatrayente (Bernhagen *et al.,* 2007), MIF puede promover el reclutamiento de macrófagos y células T al tejido adiposo, por lo que se ha implicado en la inflamación asociada a la obesidad y sus complicaciones metabólicas relacionadas.

Diversos estudios epidemiológicos relacionan los niveles sanguíneos de MIF con la obesidad. Dandona *et al.,* reportaron que los individuos con obesidad (IMC de 37.5 ± 4.9 kg/m^2) tienen concentraciones plasmáticas de MIF significativamente incrementadas (2.8 ± 2.0 ng/mL), en comparación con los individuos de peso normal con un IMC de 22.6 ± 3.4 kg/m^2 (1.2 ± 0.6 ng/mL) (Dandona *et al.,* 2004). Similarmente, los niveles séricos de MIF se encontraron incrementados en adolescentes con sobrepeso en comparación con los de peso normal (mediana: 964.6 pg/mL, rango intercuartil: 590.3-2019.46 vs. 562.7 pg/mL, rango intercuartil: 430.6-813.76), y estos se correlacionaron positivamente con marcadores de inflamación y obesidad (Kamchybekov *et al.,* 2012). Se ha demostrado que en la obesidad, el mRNA de *MIF* está sobre expresado en un 60% de las células mononucleares de sangre periférica, además el aumento en los niveles séricos y el

mRNA de *MIF* en estas células se asocian con el IMC, ácidos grasos libres y la tolerancia disminuida a la glucosa (Surk *et al.,* 2005; Vozarova *et al.,* 2002; Dandona *et al.,* 2004; Ghanim *et al.,* 2004).

Dependiendo del contexto celular y el estatus del estímulo, MIF puede unirse a diferentes receptores y desencadenar varias vías de señalización. MIF se une con alta afinidad al dominio extracelular de la proteína CD74, una proteína transmembranal tipo II. El receptor CD74 se expresa en diferentes tipos celulares como monocitos, macrófagos, células T, células B, fibroblastos, células endoteliales, epiteliales y estromales. Aproximadamente del 2-5% de CD74 se expresa en la superficie celular de monocitos (Leng *et al.,* 2003). Posteriormente, el complejo MIF/CD74 se internaliza, pero la señalización requiere el reclutamiento del co-receptor CD44 (Shi *et al.,* 2006), entonces el complejo MIF/CD74/CD44 involucra la fosforilación en serina de las colas citoplasmáticas de CD74 y CD44 y el reclutamiento de una tirosina cinasa tipo Src, llevando a la fosforilación de ERK1/ERK2 [miembros de la familia de proteínas cinasas activadas por mitógeno (MAPKs)], estos eventos llevan a la expresión de factores de transcripción como ETS (factores de transcripción que contienen dominios ETS) y AP1 (proteína activadora 1), gen del receptor tipo toll 4 (TLR4), citocinas proinflamatorias, moléculas de adhesión y metaloproteinasas. Por otro lado, también la fosforilación de ERK1/ERK2 induce a la fosfolipasa A2 (PLA2), ácido araquidónico, cinasa terminal-N JUN (JNK) y la cicloxigenasa 2 (COX2), que inhiben la apoptosis mediada por p53. Además, MIF inhibe la síntesis del inhibidor de NFκB (IκB) mediada por glucocorticoides y lleva a la activación de genes de citocinas proinflamatorias, tales como TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-8 y MIF. Una de las funciones de MIF es la capacidad de reclutar células de la repuesta inmune innata y adaptativa al sitio de la inflamación, y como ya se sabe, en la obesidad hay infiltración de macrófagos y células T al tejido adiposo, esto amplifica la producción de citocinas proinflamatorias como TNF-α, IFN-γ, IL-1β y MIF, por lo que MIF puede funcionar como un regulador directo del metabolismo de la energía, y puede afectar la adiposidad como una citocina proinflamatoria, lo que sugiere que MIF contribuye a la obesidad, a través de la regulación de la producción de citocinas inflamatorias en el tejido adiposo, mediada por su vía de señalización

(Leng *et al.,* 2003; Asare *et al.,* 2013; Tillmann *et al.,* 2013; Bucala, I-1). Para regular el tráfico de leucocitos así como también la activación de células T, MIF puede unirse a los receptores CXCR2 o CXCR4, los cuales forman complejos con CD74 (Figura 1) (Bernhagen *et al.,* 2007).
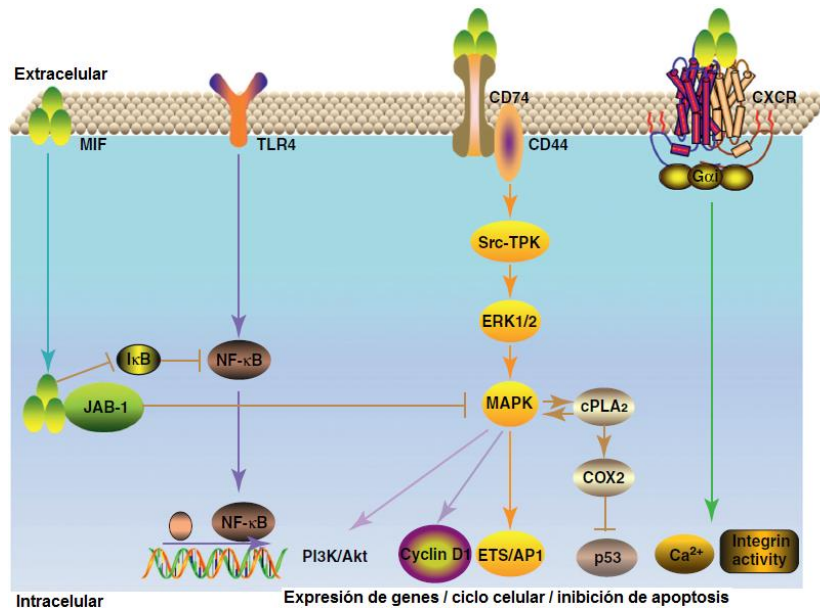

Figura 1. Vía de señalización de MIF. MIF puede inducir la cascada de señalización a través de sus receptores CD74, CXCR2 y CXCR4. Estas vías son la base para las funciones biológicas de MIF como inducción de la expresión de genes proinflamatorios, proliferación celular, inhibición de apoptosis y activación de integrinas leucocitarias (Xu *et al.,* 2013).

El gen de *MIF* humano se encuentra situado en el brazo largo del cromosoma 22 (22q11.23), y fue relacionado a una acumulación de grasa subcutánea abdominal en caucásicos, en un análisis de ligamiento de todo el genoma, aunque este hallazgo no ha sido replicado en otras poblaciones, es posible que la región 22q11.23 sea un *locus* de susceptibilidad para la adiposidad abdominal en una población en particular (Rice *et al.,* 2002; Sakaue *et al.,* 2006; Nishihira, 2012). Este gen está formado por la región promotora, 3 exones de 205, 173 y 183 pb, separados por dos intrones de 189 y 95 pb. En este gen se han descrito varios polimorfismos, en la región promotora el polimorfismo de un solo nucleótido (SNP) -173 G>C y la repetición corta en tándem (STR) -794 $CATT_{5-8}$, en intrones los SNP +254 T>C y +656 C>G (Figura 2) (Donn, 2004; Renner *et al.,* 2005; Grieb *et al.,* 2010).
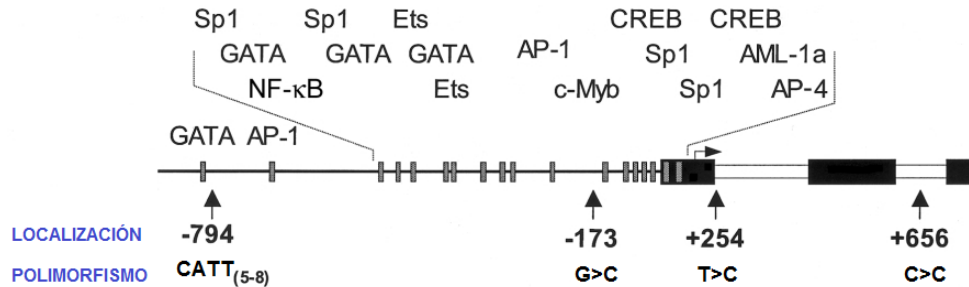
Figura 2. Representación esquemática del gen de MIF. Los 3 exones, 2 intrones y sitios de unión a factores de transcripción putativos están representados por cajas negras, blancas y grises, respectivamente. Las flechas indican las posiciones de 3 polimorfismos de un solo nucleótido y del tetranucleótido microsatélite CATT (5-8) (Renner *et al.,* 2005).

Dos polimorfismos en la región promotora del gen *MIF* con importancia funcional han sido ampliamente estudiados: el STR -794 $CATT_{5-8}$ y el SNP -173 G>C, los cuales se han relacionado con la expresión del mRNA y los niveles circulantes de MIF (Baugh *et al.,* 2002; Donn *et al.,* 2002; Radstake *et al.,* 2005). Donn *et al.*, en el 2002, reportaron que individuos con el alelo -173C presentan niveles elevados de MIF comparado con los portadores del genotipo G/G (p=0.04). En otro estudio realizado por Radstake *et al.,* en el 2005 encontraron que los portadores de los alelos 7-CATT y -173C presentaron mayores niveles de MIF (p=0.002 y p=0.004, respectivamente) comparado con aquellos que no tenían ninguno de los alelos que confieren riesgo (Donn *et al.,* 2002; Radstake *et al.,* 2005).

También se han realizado estudios in *vitro* de ambos polimorfismos, para conocer el mecanismo por el cual afectan la actividad del promotor. En el 2002, Baugh *et al.,* realizaron un estudio in *vitro* en un ensayo con el gen reportero de luciferasa en células Cos-7, A549 y CCD-19Lu y observaron que la actividad del promotor de *MIF* basal e inducida por la forskolina fue significativamente menor en células transfectadas con las construcciones que tenían el alelo 5-CATT en la posición -794, que en aquellas que tenían los alelos 6, 7 o 8-CATT. Otro estudio in *vitro* realizado por Donn *et al.,* en el 2002 utilizando un gen reportero de luciferasa en linfoblastos T humanos (CEMC7A) y células epiteliales de pulmón humano A549*,* observaron una mayor actividad del promotor cuando se encontraba el alelo -173C comparado con el alelo -173G en las células CEMC7A, en contraste a lo observado en células A549

donde la actividad del promotor fue mayor cuando se encontraba el alelo -173G, lo que sugiere que el polimorfismo -173 G>C puede afectar la actividad del promotor en una manera específica del tipo celular; además propusieron que la presencia de citosina en la posición -173 crea un sitio de unión para el factor de transcripción AP-4 (proteína activadora 4) (Donn *et al.,* 2002).

En cuanto a los polimorfismos $-794CATT_{5-8}$ y -173 G>C también se han asociado con el desarrollo de enfermedades crónico degenerativas e inflamatorias, como AR, cáncer y obesidad. Baugh *et al.,* en el 2002 encontraron que el alelo 5-CATT en la posición -794 se asoció con una baja severidad de la AR (p=0.025). En población caucásica de Reino Unido, reportaron que el alelo -173C se asocia con un mayor riesgo de desarrollar artritis idiopática juvenil (OR= 1.9 IC95% 1.4-2.7, p= 0.0002). En otro estudio realizado por Makhija *et al.,* en el 2007, encontraron que el alelo -173C se asocia con pancreatitis aguda (p=0.025). Vera *et al.,* en el 2011 realizaron un meta-análisis de estudios sobre el cáncer y el polimorfismo -173 G>C, y reportaron que el alelo -173C se asocia con un alto riesgo para desarrollar cáncer, particularmente para tumores sólidos (OR= 1.89, IC95% 1.15-3-11, p=0.012) (Baugh *et al.,* 2002; Donn *et al.,* 2002; Makhija *et al.,* 2007; Vera *et al.,* 2011). En el 2006, Sakaue *et al.,* realizaron un estudio en individuos japoneses con peso normal y obesos, y reportaron que el polimorfismo $-794CATT_{5-8}$ fue asociado con obesidad, mientras que el otro polimorfismo -173 G>C no se asoció con obesidad, así mismo analizaron por haplotipos de ambos polimorfismos y encontraron que el haplotipo G/6-CATT de los dos polimorfismos se asocia con obesidad (p= 0.028). Otro estudio realizado por Lehmann *et al.,* en el 2009 en población alemana reportaron que el haplotipo C/7-CATT se asocia con la menor supervivencia de pacientes con sepsis severa (OR=1.806, IC95% 1.337-2.439; p= 0.0005) (Sakaue *et al.,* 2006; Lehmann *et al.,* 2009).

Considerando los antecedentes mencionados, se conoce que la inflamación crónica de grado bajo que acompaña a la obesidad puede ser uno de los mecanismos que contribuye al desarrollo de RI, homeostasis de la glucosa alterada y a sus

comorbilidades tales como DMT2, aterosclerosis y enfermedades cardiovasculares. MIF es una citocina proinflamatoria involucrada en enfermedades autoinmunes e inflamatorias. Diversos estudios muestran niveles séricos incrementados de MIF en individuos obesos, con glucosa alterada y DMT2, sugiriendo que esta citocina puede contribuir a la patogénesis de la obesidad, así como a sus comorbilidades (Matia-García *et al.,* 2014). Por el papel que desempeña la inflamación subclínica en la obesidad y sus comorbilidades, el objetivo de este trabajo fue caracterizar el estado inflamatorio en jóvenes guerrerenses con y sin obesidad, determinando los subtipos de linfocitos Th, de monocitos-macrófagos, niveles de citocinas, así como la relación de variantes en el gen *MIF* con su expresión de mRNA y niveles circulantes de la proteína, con la finalidad de proponer marcadores de inflamación que puedan predecir de manera temprana la aparición de comorbilidades en los jóvenes obesos. Para abordar este planteamiento, el trabajo se ha dividido en tres capítulos que se describen a continuación:

CAPÍTULO I

Increase of Th1 cells and inflammatory monocyte-macrophages in obese young subjects

Increase of Th1 cells and inflammatory monocyte-macrophages in obese young subjects.

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Abstract

Obesity is associated with a low-grade inflammation resulting from chronic activation of the innate immune system that can subsequently lead to the development of chronic diseases. Few studies have reported the in vivo relationship of circulating T-helper cells and monocyte-macrophages with obesity, thus the importance to determine this potential association in obese young subjects lacking other concomitant diseases. By flow cytometry we assessed peripheral blood frequencies of Th1- and Th2-type cells (based on their cytokine patterns), and monocyte-macrophage subsets. Our data indicate the predominance of circulating Th1 cells in obese subjects, and the increase in the $CD68^+CD14^-$, $CD16^+CD14^-$ and $CD16^{low}CD14^-$ monocyte-macrophage subpopulations. The count of both cell types positively correlated with weight, body mass index, waist circumference and waist-hip-ratio. These results suggest that monocyte-macrophages and T cells from peripheral blood of obese subjects have a proinflammatory phenotype, which seems associated with body adiposity measures.

1. Introduction

In industrialized countries, obesity has emerged as a world health challenge, resulting in an increasing prevalence of comorbidities associated to obesity. Several studies have found that obesity, and increased adiposity mainly in the abdominal region, are associated with insulin resistance (IR), impaired glucose homeostasis and comorbidities such as type 2 diabetes mellitus (T2D) and cardiovascular disease [1-3]. Adipose tissue (AT) is now considered to be an active endocrine organ that secretes important mediators of inflammation, and its shift to deregulated production of pro-inflammatory cytokines over the anti-inflammatory cytokines in obesity contributes to the low-grade chronic

16

inflammation, which is recognized as an important factor to the pathogenesis of obesity complications [2, 4].

Low-grade inflammation in obesity and T2D is considered the product of an activated innate immune system, which triggers further infiltration of inflammatory cells, such as macrophages, mast cells, neutrophils, dendritic cells and lymphocytes, resulting in qualitative and quantitative alterations of the stromal vascular fraction of the white AT [5-7]. The contribution of the adaptive immune system in obesity, particularly T-cells has not been sufficiently studied; however, experimental evidence indicating that lymphocytes are important in obese white AT inflammation is emerging. Alteration in the balance between different T-cell populations within visceral adipose tissue in particular appears to be an early event in obesity. Defined by their cytokine profile, $CD4^+$ T helper cells can differentiate into a pro-inflammatory type 1 (Th1) or Th1-repressing type 2 (Th2) [8-10].

In 2009, three separate studies demonstrated the key role of T cells infiltrating the white AT for the development of insulin resistance and the associated inflammatory phenotype in genetically obese mouse models [11-13]. Several studies have revealed that, in obese mice and men, visceral white AT contains more T cells, and this is likely due to increased T cell expansion or recruitment [14-16]. Recent data indicate that obesity is characterized by increased activation of circulating immune cells from both the innate and adaptive immune system. In subjects with moderate or severe obesity after a hypocaloric diet or gastric surgery, weight loss led to a diminution of the $CD14^+CD16^+$ and $CD14^{dim}CD16^+$ monocyte subsets [17]. Viardot et al., reported in morbidly obese subjects with T2D that caloric restriction with modest weight loss markedly reduces pro-inflammatory Th1-cell numbers, with little effect on Th2 cell numbers [18]; they also reported increased T-cell expression of the activation marker CD25 (interleukin-2 receptor) in obese subjects [19]. These findings

suggest that the systemic inflammation found in obesity may include an over-activated adaptive immune system.

The aim of this study was to characterize the phenotype of circulating T helper cells and monocyte-macrophages and their relationship to obesity in young subjects without related diseases.

2. Materials and Methods

2.1. Subjects. We recruited a total of 100 subjects, 18 to 30 years old, from the state of Guerrero, Mexico. Participants were not under any medication. All subjects gave written informed consent prior to the study. This protocol was approved by the Research Ethics Committee of the Autonomous University of Guerrero.

2.2. Anthropometric and clinical measurements. Body weight was determined in light clothes and without shoes, using a body composition monitor (Tanita TBF-300 GS), and the height was measured to the nearest 0.1 cm using a stadiometer (Seca, Hamburg, Germany). From these measurements, BMI was calculated (BMI = weight/height^2, kg/m^2). 50 subjects were classified in the obese group (BMI $\geq$30 kg/m^2) and 50 subjects in normal-weight group (BMI 18.5 to 24.9 kg/m^2), according to the criteria of the World Health Organization [20].

The body circumferences were measured with an anthropometric tape accurate to within $\pm$0.1 cm (Seca 201, Hamburg, Germany). Blood pressure was measured in the sitting position with an automatic sphygmomanometer (OMRON) on the left arm after 10 min rest. The systolic blood pressure (SBP) and diastolic blood pressure (DBP) were calculated from two readings with a minimal interval of 10 min.

2.3. Biochemical variables. A fasting blood sample was taken to measure total cholesterol, HDL-cholesterol (HDL-c), LDL-cholesterol (LDL-c), triglycerides (TG) and glucose levels, these parameters were determined with commercially available kits (Spinreact). Leukocyte, erythrocyte and platelet counts were assessed by an automated analyzer (BC-2800, Mindray).

2.4. Peripheral blood mononuclear cells isolation and flow cytometry analysis. Peripheral blood mononuclear cells (PBMCs) were isolated from ethylenediaminetetraacetic acid (EDTA) blood samples by Ficoll-Hypaque™ Premium (GE Healhtcare) density gradient centrifugation and were counted by trypan blue exclusion for each subject. Cell samples from all studied subjects were individually manipulated and analyzed by four-color flow cytometry in a FACSCanto II flow cytometer (BD Biosciences), after staining with fluorochrome-conjugated antibodies to cell surface markers of T helper cells and monocyte-macrophages: anti-CD3-APC (clone UCHT1), anti-CD4-FITC (clone RPA-T4), anti-CD14-FITC (cloneHCD14), anti-CD16-PE/Cy7 (clone 3G8), anti-CD68-PE (clone Y1/82A), anti-CD64-PE/Cy7 (clone 10.1), anti-CD86-APC (clone IT2.2), anti-CD163-APC (clone GHI/61), anti-CD206-PE (clone 15-2); all were purchased from BioLegend.

Th1/Th2 helper T cells were quantified by intracellular cytokine staining for anti-IFN-ɣ-PE (clone B27) (Th1) and anti-IL-4-PE (clone 8D4-8) (Th2) (BioLegend): PBMCs were incubated with Brefeldin-A (BFA, BioLegend) for 5 h at 37°C. After surface staining for CD3 and CD4 (to select the CD3$^+$/CD4$^+$ T helper cells), cell were fixed and permeabilized using BD Cytofix/Cytoperm™, and BD Perm/Wash™ (BD Bioscience, Pharmingen) according to the manufacter´s instructions. After intracellular staining for IFN-ɣ and IL-4, cells were immediately analyzed by flow cytometry, calculating the percentage and absolute number of Th1 and Th2 cells of the total CD3$^+$CD4$^+$ population. To determine

monocyte-macrophages, the cells were incubated with anti-human CD14, CD16, CD68, CD64, CD86, CD163 and CD206 for 30 minutes at 4°C and immediately analyzed by flow cytometry. Analysis of flow cytometry data was performed using the FlowJo version 7.6.1 software. The immune cell subsets were expressed as a percentage and absolute number of the total white cell count.

2.5. Statistical analysis. Data analysis was performed using STATA software (v.11.0) and GraphPad Prism (v 5.0). Differences in characteristics between groups were analyzed using the chi-square test for categorical variables (data presented as percentages), Student's *t*-test for parametric variables (data presented as mean ± SD) and Mann-Whitney *U*-test for non-parametric variables (data presented as median and 5th to 95th percentiles). Correlations between variables were expressed as Spearman's correlation coefficients. P < 0.05 was considered statistically significant.

3. Results

3.1. Anthropometric and biochemical parameters. Anthropometric and biochemical characteristics of the study subjects are summarized in Table 1. As expected, obese subjects had higher body weight, BMI, waist circumference, hip circumference and waist-hip-ratio, as well as elevated systolic and diastolic blood pressures, triglycerides levels, prevalence of hypertension and leukocyte counts higher than in normal-weight subjects (P <0.05). There were no significant differences by age, gender, glucose, cholesterol, LDL-c and HDL-c between groups (P > 0.05).

3.2. Th1 and Th2 helper T cells in obese subjects. The combination of CD3 and CD4 staining of T-helper lymphocytes in PBMCs from the subjects with normal-weight and

obesity, revealed the existence of six subpopulations of T cells: $CD3^+CD4^+$, $CD3^+CD4^-$, $CD3^+CD4^{int}$, $CD3^-CD4^{int}$, $CD3^{int}CD4^-$, $CD3^{int}CD4^{int}$. Obese subjects had a higher number of $CD3^+CD4^+$ ($P=0.036$), $CD3^-CD4^{int}$ ($P=0.0002$) and $CD3^{int}CD4^{int}$ ($P=0.0013$) T cells than in normal-weight subjects (Figures 1a and 1c).

$CD3^+CD4^+$ T-helper cell subpopulations were further characterized for Th1 and Th2 phenotypes, determined by their intracellular cytokine profile measuring IFN-ɣ and IL-4, respectively. With no stimulation, the production of IFN-ɣ by $CD3^+CD4^+$ cells was low, as expected. However, a substantial difference in Th1 cell numbers was recorded between normal-weight and obese individuals. Th1 cell number of the subpopulations $CD3^+CD4^+IFN\text{-}ɣ^+$, $CD3^{int}CD4^-IFN\text{-}ɣ^+$ and $CD3^{int}CD4^{int}IFN\text{-}ɣ^+$ was increased in obese group compared with normal-weight group: 2285 vs 1276 cells/mL, $P<0.01$; 846 vs 640 cells/mL, $P<0.05$ and 1018 vs 639 cells/mL, $P<0.01$ (Figures 1a and 1d). Conversely, $CD3^+CD4intIL\text{-}4^+$ Th2 cell number were lower in obese group than in normal-weight group (8294 vs 13529 cells/mL, $P<0.05$) (Figures 1b and 1e). Th2 cells percentage was compared between groups, the obese subjects showed a lower percentage of cells $C3^+CD4^+IL4^+$ (2.43 vs 2.72 %, $P<0.05$), $C3^+CD4^{int}IL4^+$ (0.32 vs 0.62 %, $P<0.001$), $C3^+CD4^-IL4^+$ (1.26 vs 1.94 %, $P<0.001$) and $C3^{int}CD4^-IL4^+$ (0.15 vs 0.27 %, $P<0.05$) compared with normal-weight group). Similar findings were observed with mean fluorescence intensity (MFI) for the expression of IFN-ɣ (Th1) and IL-4 (Th2) in $CD3^+CD4^+$ T-helper cell subpopulations. Obese subjects show an increased expression of IFN-ɣ in $CD3^+CD4^{int}IFNɣ^+$ cells (364 vs 324, $P<0.05$) and in $CD3^{int}CD4^{int}IFNɣ^+$ cells (881 vs 664, $P<0.01$) compared with normal-weight subjects (Figure 2a). Conversely, the expression of IL-4 in the $CD3^+CD4^-IL4^+$ cells (732 vs 985, $P<0.05$), in the $CD3^-CD4^{int}IL4^+$ (4474 vs 7605, $P<0.001$), the $CD3^{int}CD4^-IL4^+$

(2181 *vs* 3197, $P<0.01$) and the CD3^{int}CD4^{int}IL4^{+} population (3901 *vs* 6644, $P<0.001$) in obese subjects decreases compared with normal-weight group (Figure 2b).

Furthermore, when comparing the number and MFI of T cells according to the IMC categorized (18.5-24.9, 30-34.9 and $\geq$35 kg/m^2), it was found that those with BMI of 30-34.9 kg/m^2 have a greater number of T cells regarding those with BMI of 18.5-24.9 kg/m^2. However, in those with a BMI $\geq$35 kg/m^2 there were not significant differences (data not shown).

3.3. Monocyte-macrophage distribution in obese and normal-weight subjects. We quantified monocyte-macrophage in PBMCs of normal-weight and obese subjects by four-color flow cytometry. In separate experiments PBMCs were stained with CD14, CD68, CD64, CD86, CD16, CD163 and CD206. First, we observed three monocyte-macrophage subpopulations, according to CD14 and CD68: CD68^{+}CD14^{-}, CD68^{int}CD14^{hi}, CD68^{low}CD14^{int}. Obese subjects had a higher number of CD68^{+}CD14^{-} cells than normal-weight subjects (289 *vs* 171 cells/mL, $P<0.01$); but the count of all other cell populations were similar among groups (Figures 3a and 3b). Monocyte-macrophage subpopulations were further characterized for CD64 and CD86 expression. We found no significant differences in the counts of monocyte-macrophage subpopulations: CD68^{int}CD14^{hi}CD64^{+}CD86^{+}, CD68^{low}CD14^{int}CD64^{+}CD86^{+} and CD68^{low}CD14^{int}CD64^{hi}CD86^{+} in subjects with and without obesity (Figures 3a and 3c). We then analyzed the number of CD68^{+}CD14^{-} monocyte-macrophage by gender. Obese women had a higher number of CD68^{+}CD14^{-} cells (275 *vs* 153 cells/mL, $P<0.01$) than normal-weight women. Similarly, obese men had a higher number of CD68^{+}CD14^{-} cells

(290 vs 209 cells/mL, $P=0.33$) than normal-weight men, but this difference was not statistically significant (Figure 3d). From the staining of PBMCs with CD14 and CD16, we observed 4 subpopulations of monocyte-macrophage cells: $CD16^+CD14^-$, $CD16^{int}CD14^+$, $CD16^{int}CD14^{hi}$ and $CD16^{low}CD14^-$. The number of the subset $CD16^+CD14^-$ ($P<0.01$) and $CD16^{low}CD14^-$ ($P<0.01$) was higher in obese subjects than in the normal-weight group (Figures 4a and 4b). $CD16^+CD14^+$ monocyte-macrophage subpopulations were also stained with CD163 and CD206. There was no difference in the number of cells of the different monocyte-macrophage subpopulations: $CD16^{int}CD14^+CD163^+CD206^{int}$ and $CD16^{int}CD14^{hi}CD163^+CD206^{int}$ in subjects with and without obesity (Figures 4a and 4c).

3.4. Relation of body measures with count cells of the innate and the adaptive immune system. To evaluate a potential relationship between the measures of central adiposity with the count of monocyte-macrophages and T cells, correlations were performed. In the whole population, we found that the number of $CD68^+CD14^-$ monocyte-macrophages was correlated with weight ($r=0.31$; $P=0.002$), BMI ($r=0.29$; $P=0.004$), waist circumference ($r=0.36$; $P=0.0002$) and waist-hip-ratio ($r=0.41$; $P=<0.001$). Similarly, weight, BMI, waist circumference and waist-hip-ratio were significantly correlated with the number of $CD3^+CD4^+$, $CD3^-CD4^{int}$ and $CD3^{int}CD4^{int}$ cells (Table 2).

The numbers of $CD3^+CD4^+IFNɣ^+$ and $CD3^{int}CD4^{int}IFNɣ^+$ Th1 cells and the expression of IFN-ɣ in $CD3^{int}CD4^{int}IFNɣ^+$ cells were positively correlated with the measures of central adiposity (Tables 2,4). In contrast to Th1 cells, the number and percentage of Th2 cells: $CD3^+CD4^{int}IL4^+$ cell/mL, $CD3^+CD4^+IL4^+$%, $CD3^+CD4^{int}IL4^+$%, $CD3^+CD4^-IL4^+$% and $CD3^{int}CD4^-IL4^+$% were negatively correlated with weight, BMI, waist circumference, hip circumference and waist-hip ratio. Similarly, the expression of IL-4 in $CD3^{int}CD4^+IL4^+$,

$CD3^+CD4^-IL4^+$ and in $CD3^-CD4^+IL4^+$ cells, was negatively correlated with measures of central adiposity (Tables 2-4).

4. Discussion

In this study we found that obesity seems characterized by an increase of circulating immune cells from both the innate and adaptive immune system. We report six subpopulations of T-helper cells: $CD3^+CD4^+$, $CD3^+CD4^-$, $CD3^+CD4^{int}$, $CD3^-CD4^{int}$, $CD3^{int}CD4^-$, $CD3^{int}CD4^{int}$ in PBMCs of normal-weight and obese subjects. The circulating $CD3^+CD4^+$, $CD3^-CD4^{int}$ and $CD3^{int}CD4^{int}$ T cells were increased in obese subjects regarding the normal-weight group. This finding, together with an elevated number of $CD3^+CD4^+IFN\text{-}\gamma^+$, $CD3^{int}CD4^-IFN\text{-}\gamma^+$ and $CD3^{int}CD4^{int}IFN\text{-}\gamma^+$ Th1 cells, suggests that the systemic inflammation reported in obesity includes also activated cells of the adaptive immune system.

Few studies have reported the relationship of circulating T-helper cells with obesity. In 2006, it was shown the dominance of the Th1 phenotype in children with obesity, in addition the Th1 cell number was positively correlated with insulin resistance. In contrast, for the Th2 phenotype there was no significant difference in children with and without obesity [21]. Our data confirm the predominance of Th1 cells in adults with obesity. Furthermore, we found that the phenotype of $CD3^+CD4^+IL4^+$, $CD3^+CD4^{int}IL4^+$, $CD3^+CD4^-IL4^+$ and $CD3^{int}CD4^-IL4^+$ Th2 cells was decreased in obese subjects. These results show that obesity is characterized by the circulating T cells balance polarized towards a pro-inflammatory Th1 phenotype, this suggests that the metabolic dysregulation caused by obesity can be affected by $CD3^+CD4^+$ T cells and that the imbalance between Th1 and Th2 subsets may also be a pathophysiological component of obesity.

In both humans and rodents with increased body weight, adipose tissue macrophage accumulation is directly proportional to measures of adiposity, and the content of macrophages correlates positively with insulin resistance, T2D, altered lipid metabolism and promote atherosclerosis [5-6, 22-23]. Furthermore, in diverse patient groups, circulating inflammatory mediators and activated $CD14^+$ monocytes and neutrophils have been identified as inflammatory agents linking obesity to its metabolic and cardiovascular complications [19, 24-26]. In contrast, in our study we observed an increase in the $CD68^+CD14^-$, $CD16^+CD14^-$ and $CD16^{low}CD14^-$ monocyte-macrophage subpopulations in obese subjects. Likewise, other studies have shown that obesity is characterized by increased circulating $CD16^+$, $CD14^{dim}CD16^+$ and $CD14^+CD16^+$ monocytes [17, 27-28]. A significant increase in the $CD16^+$ subset has also been described in inflammatory diseases such as sepsis, rheumatoid arthritis, and infections [29], and related to cardiovascular events in patients with chronic kidney disease [30-31]. Unlike the $CD14^+CD16^-$ monocytes that express a high level of chemokine (C-C motif) receptor (CCR)-2 (chemokine ligand-2 receptor) and low levels of CCR5 (chemokine ligand-3 receptor) and CX3 chemokine receptor-1 (the fractalkine receptor), the $CD16^+$ subpopulation is CCR2 negative but express high levels of CX3 chemokine receptor-1 and CCR5 receptors, as well as high levels of tumor necrosis factor-α (TNF-α) and lowest levels of interleukin-10, as compared with $CD16^-$ monocytes [29]. These $CD16^+$ cells show a macrophage-like phenotype with enhanced antigen-presenting capacity, high endothelial affinity and a potent ability to invade vascular lesions and the cells produce proinflammatory cytokines [28-29]. Based on these observations, we would like to propose the $CD16^+$ monocytes as early markers of low-grade inflammation in obesity and its comorbidities related.

An important finding from the present study is that obese women had an increased number of $CD68^+CD14^-$ cells compared with normal-weight women. This may indicate that the increase in these cells might be due to weight gain, because women only had obesity and any other metabolic disorder. Although could also be influenced by the gender, since it has been reported that compared with men, women have low cardiovascular risk before menopause. This protective mechanism in women is not completely understood, it may be partially related to the protective effect of endogenous estrogen on cardiovascular risk. The risk factor prevalence in female should be higher than in male to lead to the onset of cardiovascular disease at the same age as in male, since coronary heart disease typically appears 10 years later in female [32-33]. Further studies are required to establish the potential functions of the $CD68^+CD14^-$ subpopulation in the development of obesity and its associated comorbidities.

For a long time, obesity has been seen as an inflammatory disease characterized by a low-grade chronic inflammatory state, and has been associated with increased circulating concentrations of inflammatory cytokines and acute-phase proteins. It is now established that the enlarged fat mass characterizing obesity is associated also with macrophage accumulation and with alteration of adipose tissue secretions [34]. In this study we tried to unravel correlations between the distribution of body fat, with monocyte-macrophages and T cells. We found that the number of $CD68^+CD14^-$ monocyte-macrophages, $CD3^+CD4^+$, $CD3^-CD4^{int}$, $CD3^{int}CD4^{int}$, $CD3^+CD4^+IFN\text{-}\gamma^+$ and $CD3^{int}CD4^{int}IFN\gamma^+$ T cells and the expression of IFN-γ in $CD3^{int}CD4^{int}IFN\gamma^+$ cells were correlated with weight, BMI, waist circumference and waist-hip-ratio. In contrast to Th1 cells, the $CD3^+CD4^{int}IL4^+$, $CD3^+CD4^+IL4^+$, $CD3^+CD4^{int}IL4^+$, $CD3^+CD4^-IL4^+$ and $CD3^{int}CD4^-IL4^+$ Th2 cells were

negatively correlated with weight, BMI, waist circumference, hip circumference and waist-hip ratio. Similarly, the expression of IL-4 in $CD3^{int}CD4^{+}IL4^{+}$, $CD3^{+}CD4^{-}IL4^{+}$ and $CD3^{-}CD4^{+}IL4^{+}$ cells, was negatively correlated with measures of central adiposity. It has been shown that in morbidly obese subjects with T2D and prediabetes who underwent 24 weeks dietary energy restriction with modest weight loss, the reduction in T cells CD69 expression was correlated with BMI reduction, and the fall in Th1/2 ratio was correlated with the reduction in weight and waist. In the same study, a greater weight loss after surgery was correlated with lower macrophage CD11b expression in subcutaneous and visceral adipose tissue [18]. Another study conducted in 622 healthy volunteers showed that the $CD14^{+}$ $CD16^{+}$ and CD16+ cell count was correlated with BMI [28]. It has been observed that body fat distribution is an important risk factor for obesity-related diseases. Excess of intra-abdominal fat rather than subcutaneous fat (central vs. peripheral obesity) is associated with metabolic syndrome and cardiovascular disease [35]. In obese subjects, adipocytes, macrophages and epithelial cells communicate via obesity-associated hormones, inflammatory cytokines, and other mediators. Adipose tissue is known to produce and secrete various adipokines, such as leptin and adiponectin, as well as proinflammatory factors such as TNF, IL-6, IL-1, and C-reactive protein (CRP) [2, 36-37]. Several studies have demonstrated that the circulating levels of IL-6, MCP-1, IL-8, MIF and CRP were positively correlated to BMI and waist circumference [36, 38-40]. These studies support the idea that visceral fat may play a role in the elevation of proinflammatory cytokines in peripheral blood and immune cells in obesity.

A limitation of this study is that plasma levels of Th1 and Th2 cytokines were not measured; therefore the relationship of these cytokines with immune cells and body fat distribution remains uncertain in the studied population, but is something we plan to do.

In summary, we found that the obese group has increased circulating T cells that are polarized towards the Th1 pro-inflammatory phenotype and also an increase of $CD68^+CD14^-$ and $CD16^+CD14^-$ monocytes-macrophages, compared with normal-weight subjects. On the other hand, anti-inflammatory Th2 cells are decreased in obese subjects. We also found that the number of pro-inflammatory cells increases in subjects with a BMI of 35 kg/m^2, but this effect was not observed when the BMI is greater than 35 kg/m^2. Another studies found that Th1 cells are decreased in young subjects, either by exercise or by some other methods of intervention that lead to weight loss. Previous studies in morbidly obese subjects with T2D and prediabetes show that caloric restriction with modest weight loss reduces pro-inflammatory Th1-cell numbers, shifting the Th1 to Th2 ratio toward an anti-inflammatory Th2 phenotype, and a lower activation of adipose tissue macrophage in subcutaneous and visceral adipose tissue. Morbid obesity is characterized by circulating activated immune cells and insulin resistant, with the T-cell balance polarized towards a pro-inflammatory Th1 phenotype [18-19]. In other study in subjects with moderate or severe obesity after a hypocaloric diet or gastric surgery, weight loss reduces the $CD14^+CD16^+$ and $CD14^{dim}CD16^+$ monocyte subsets [17]. The mechanisms by which energy restriction and weight loss decrease pro-inflammatory immune cells in obesity are unclear, but some of them could be functional changes in adipose tissue biology, homeostasis of the immune cells and circulating cytokines or distinct effects of energy restriction, independent of weight loss [18].

In conclusion, we provide evidence that obese young subjects have increased Th1 cells and inflammatory monocyte-macrophages in peripheral blood, which may be used as early markers of obesity comorbidities.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Tables and figures

Table 1: Clinical and biochemical characteristics of the study subjects.

| Variables	Total (n=100)	Normal-weight (n=50)	Obesity (n=50)	P value
Age (years)^c	21(18-28)	20(18-28)	22(18-28)	0.15
Gender n(%) ^{a}				0.69
Male	46(46)	24(48)	22(44)	
Female	54(54)	26(52)	28(56)	
Weight (kg)^c	76.9(48-107.2)	59.7(43.1-73)	89.3(78.5-109)	<0.001
Height (cm)^b	164±8	164±8.4	165± 7.6	0.54
BMI (kg/m^2)^c	27.7(19.4-37.5)	22.4(18.7-24.6)	33.4(30-38.8)	<0.001
Waist circumference (cm)^c	89.5(72-115)	79.3(70.5-89)	104(90-120.5)	<0.001
Hip circumference (cm)^c	105(89.5-125)	96(87-104)	115.3(106-131)	<0.001
Waist-hip-ratio^b	0.87± 0.07	0.83± 0.05	0.9±0.07	<0.001
SBP (mmHg)^c	112(95-137)	107(92-130)	117(102-141)	<0.001
DBP (mmHg)^b	73± 9.5	69.6± 8.5	76±9.4	0.0005
Hypertension n(%)^a				0.003
No	83(83)	47(94)	36(72)	
Yes	17(17)	3(6)	14(28)	
Glucose (mg/dL)^c	86(73-105.5)	84.5(73-104)	87.5(76-107)	0.06
Cholesterol (mg/dL)^b	161±30.5	157±29.6	166±31	0.13
Triglycerides (mg/dL)^c	108(43-218.5)	84(42-188)	119(43-358)	0.002
LDL-C (mg/dL)^c	106(69-200.5)	109(69-207)	102(69-187)	0.42
HDL-C (mg/dL)^c	39.5(27.5-64)	40.5(28-68)	39(27-62)	0.68
Platelets (10^3/mm^3)^b	226±44	222±37	230±50	0.36
Erythrocytes (10^6/mm^3)^b	4.9±0.5	4.9±0.5	4.9±0.4	0.89
Leukocytes (10^3/mm^3)^b	7.1± 1.6	6.7± 1.4	7.4±1.6	0.022

BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; LDL-C, Low Density Lipoprotein-Cholesterol; HDL-C, High Density Lipoprotein-Cholesterol. [a] Data presented as n and percentage. Chi-square test. [b] Data presented as mean±SD. Student *t*-test. [c] Data presented as median and 5th and 95th percentile. Mann-Whitney test.
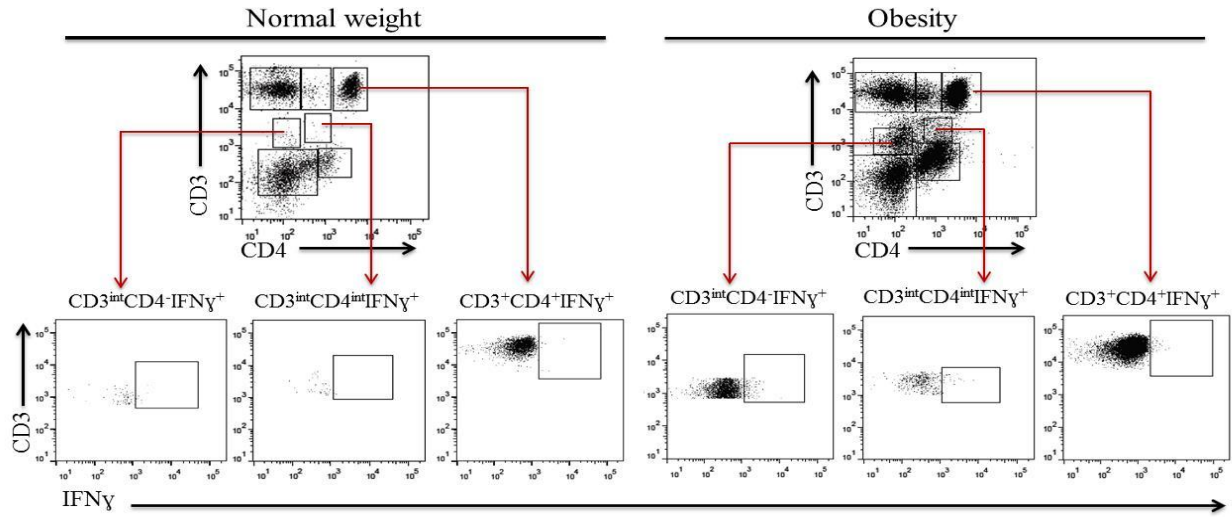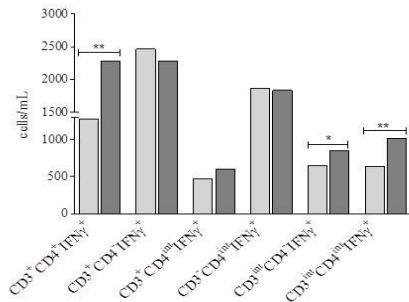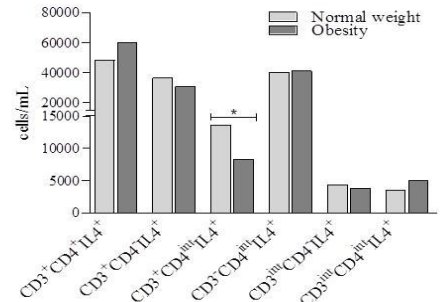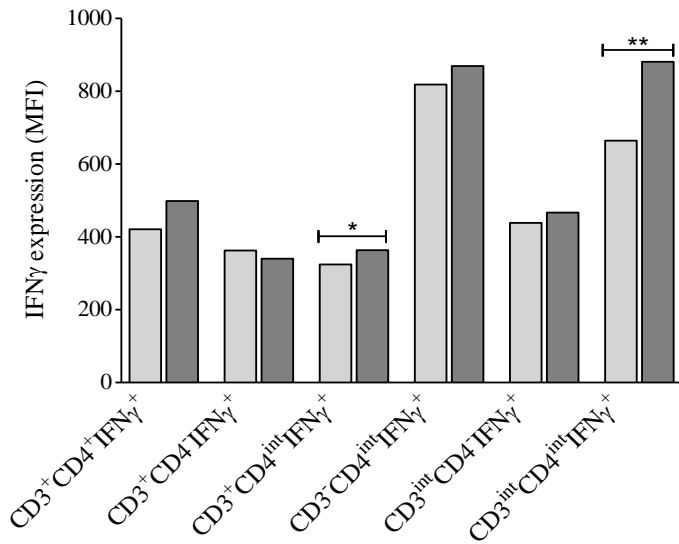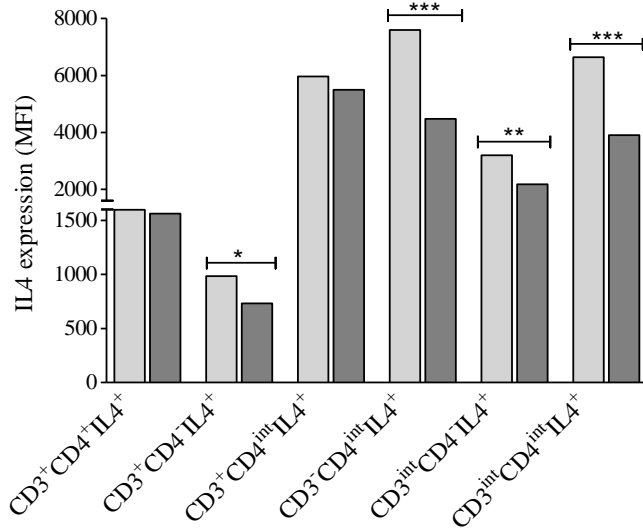
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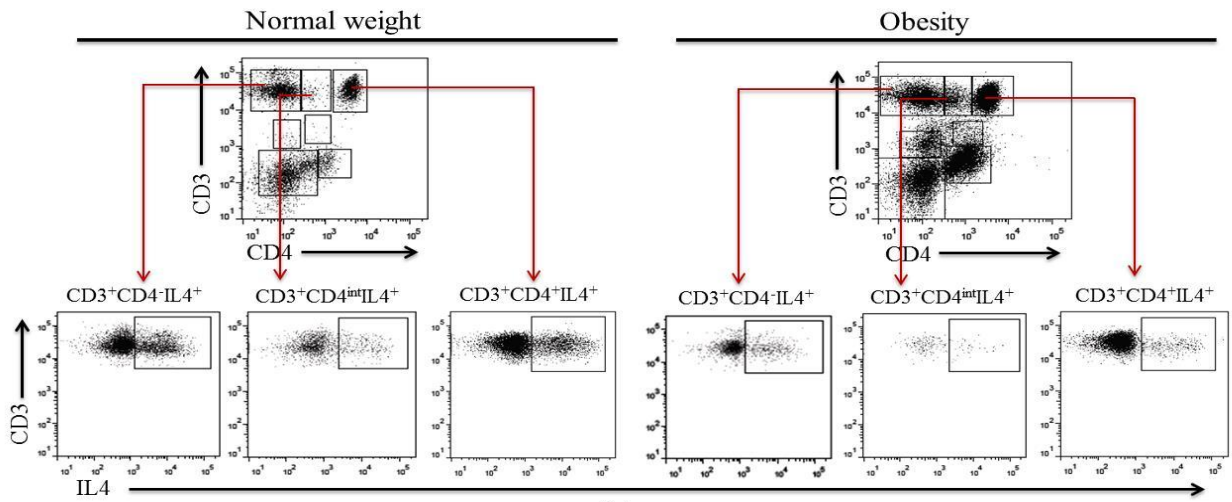
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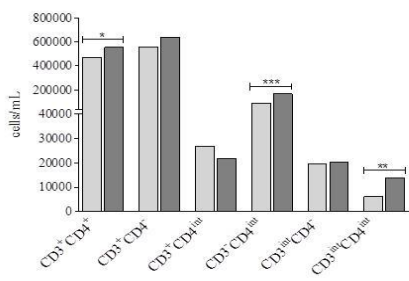
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Figure 1: Comparative analysis of CD3$^+$CD4$^+$ T-helper subpopulations in peripheral blood mononuclear cells in subjects with and without obesity. (a, c) Quantification of circulating T cells based on the expression of CD3 and CD4, we observed six T cells subpopulations: CD3$^+$CD4$^+$, CD3$^+$CD4$^-$, CD3$^+$CD4^{int}, CD3$^-$CD4^{int}, CD3^{int}CD4$^-$, CD3^{int}CD4^{int}. Based on the intracellular IFN-ɣ and IL-4, the CD3$^+$CD4$^+$ T-helper subpopulations were quantified as Th1 cells (a, d) and Th2 (b, e), expressed as absolute subset counts of the total CD3$^+$CD4$^+$ population. All values are expressed as median and 5th and 95th percentile. Comparison among groups was performed using Mann-Whitney test. *P<0.05, **P<0.01, ***P<0.001

(a)

(b)

Normal weight
Obesity

Figure 2: Comparative analysis of IFN-ɣ and IL-4 expression in different CD3^{+}CD4^{+} T-helper cell subpopulations in normal-weight and obese subjects. (a) Mean fluorescence intensity (MFI) of IFN-ɣ (Th1) and (b) IL-4 (Th2). All values are expressed as median and 5th and 95th percentile. Comparison among groups was performed using Mann-Whitney test. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

(a)

(b)

(c)
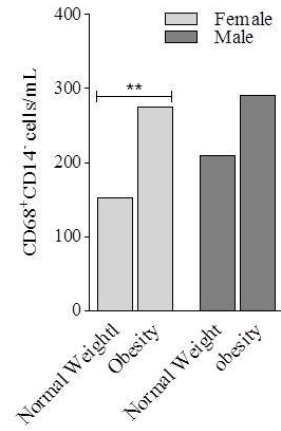
(d)

Figure 3: Characterization of monocyte-macrophage subpopulations in peripheral blood mononuclear cells in subjects with and without obesity. (a, b) Quantification of circulating monocyte-macrophage based on the expression of CD68 and CD14. We observed 3 monocyte-macrophage subpopulations: CD68+CD14-, CD68intCD4hi, and CD68lowCD14int. (a, c) CD68CD14 monocyte-macrophage subpopulations were further characterized for CD64 and CD86. (d) We analyzed the number of CD68+ CD14- monocytes and macrophages by gender. All values are expressed as median and 5th and 95th percentile. Comparison among groups was performed using Mann-Whitney test.*P<0.05, **P<0.01, ***P<0.001.

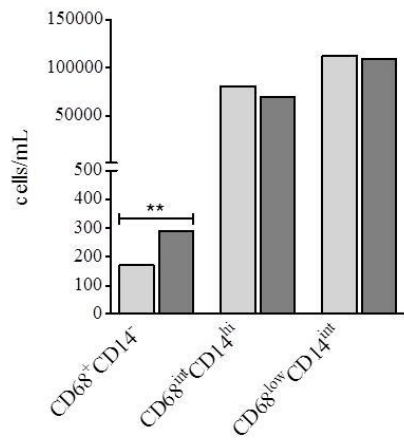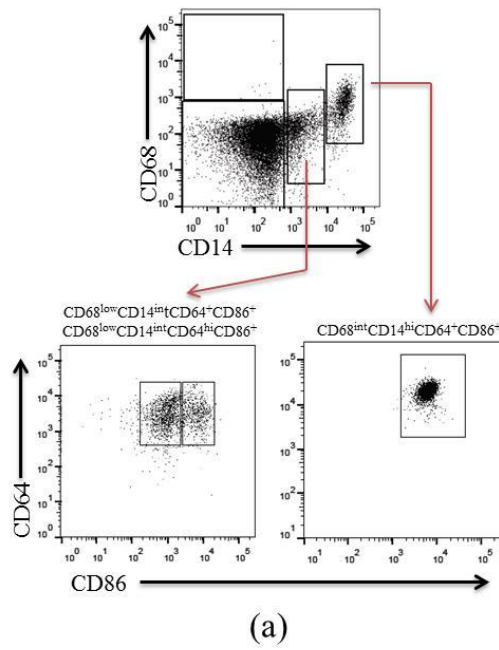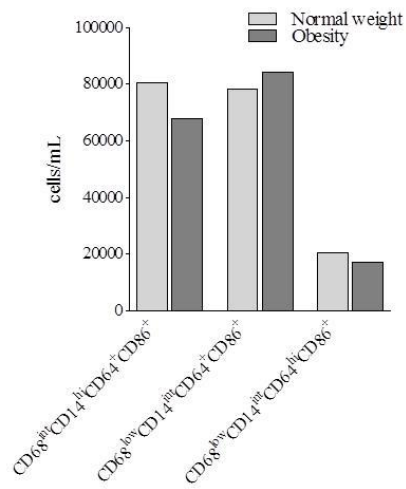CD14+CD16intCD163+CD206int   CD14hiCD16intCD163+CD206int
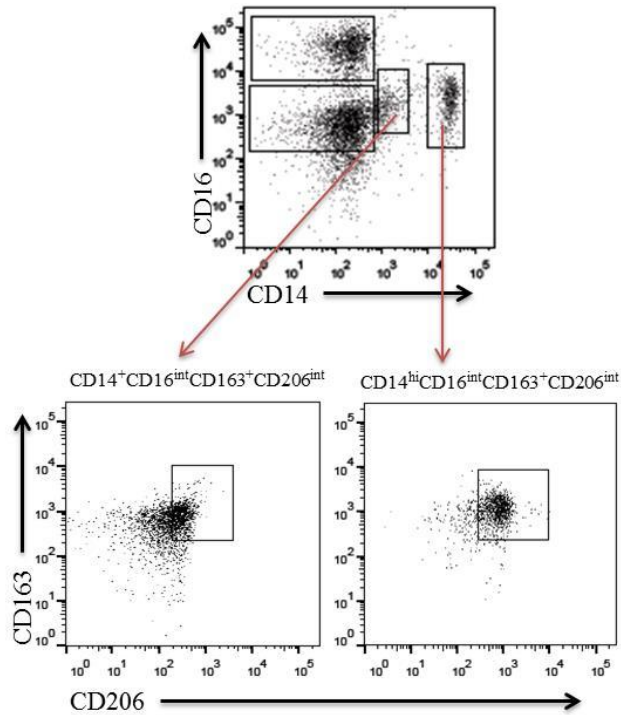
(a)
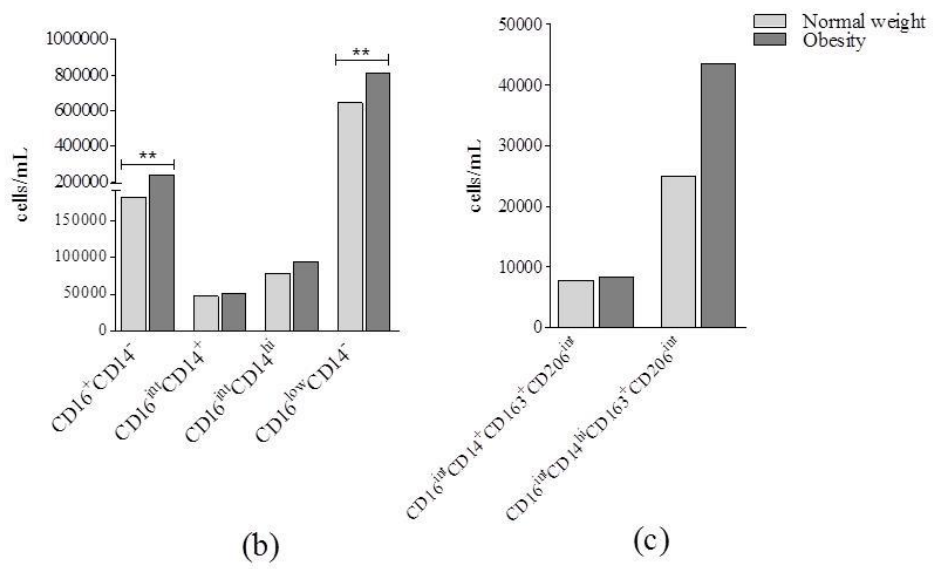
(b)

(c)

Figure 4: Characterization of monocyte-macrophage subpopulations in peripheral blood mononuclear cells in subjects with and without obesity. (a, b) Quantification of circulating monocyte-macrophage based on the expression of CD16 and CD14, we observed 4 monocyte-macrophage subpopulations: CD16+CD14-, CD16intCD14+, CD16intCD14hi and CD168lowCD14-. (a, c) CD16CD14 monocyte-macrophage subpopulations were further characterized for CD163 and CD206. All values are expressed as median and 5th and 95th percentile. Comparison among groups was performed using Mann-Whitney test.*P<0.05, **P<0.01, ***P<0.001.

Table 2: Correlation between the number of cells/mL of cell subpopulations and anthropometric measures.

Variables	CD68$^+$CD14$^-$		CD3$^+$CD4$^+$		CD3$^-$CD4^{int}		CD3^{int}CD4^{int}		CD3$^+$CD4$^+$IFNγ$^+$		CD3^{int}CD4^{int}IFNγ$^+$		CD3$^+$CD4^{int}IL4$^+$	
	r	P	r	P	r	P	r	P	r	P	r	P	r	P
Weight	0.31	0.002	0.23	0.02	0.35	0.0004	0.29	0.004	0.25	0.012	0.22	0.03	-0.10	0.32
BMI	0.29	0.004	0.20	0.04	0.34	0.0007	0.32	0.001	0.31	0.002	0.25	0.012	-0.16	0.10
Waist circumference	0.36	0.0002	0.23	0.02	0.39	0.0001	0.35	0.0003	0.32	0.001	0.31	0.002	-0.19	0.06
Hip circumference	0.18	0.07	0.21	0.03	0.28	0.005	0.29	0.004	0.29	0.003	0.21	0.04	-0.14	0.17
Waist-hip-ratio	0.41	<0.001	0.18	0.07	0.41	<0.001	0.32	0.002	0.25	0.012	0.34	0.0006	-0.22	0.02

r= Spearman correlation coefficient; P = p value; BMI, body mass index.

Table 3: Correlations between % T cell subpopulations and anthropometric measures.

Variables	CD3$^+$CD4$^+$IL4$^+$		CD3$^+$CD4^{int}IL4$^+$		CD3$^+$CD4$^-$IL4$^+$		CD3^{int}CD4$^-$IL4$^+$	
	r	P	r	P	r	P	r	P
Weight	-0.17	0.08	-0.28	0.004	-0.34	0.007	-0.23	0.024
BMI	-0.11	0.26	-0.31	0.002	-0.30	0.002	-0.23	0.023
Waist circumference	-0.19	0.05	-0.36	0.0003	-0.38	0.001	-0.23	0.02
Hip circumference	-0.10	0.36	-0.29	0.004	-0.28	0.004	-0.18	0.06
Waist-hip-ratio	-0.24	0.016	-0.34	0.0006	-0.34	0.005	-0.20	0.04

r= Spearman correlation coefficient; P = p value; BMI, body mass index.

Table 4: Correlations between the MFI of T cell subpopulations and anthropometric measures.

Variables	CD3^{int}CD4^{int}IFNγ$^+$		CD3^{int}CD4$^+$IL4$^+$		CD3$^+$CD4$^-$IL4$^+$		CD3$^-$CD4$^+$IL4$^+$	
	r	P	r	P	r	P	r	P
Weight	0.23	0.02	-0.31	0.002	-0.27	0.008	-0.37	0.002
BMI	0.25	0.012	-0.32	0.001	-0.23	0.024	-0.39	0.001
Waist Circumference	0.31	0.002	-0.38	0.0001	-0.28	0.004	-0.46	<0.001
Hip Circumference	0.19	0.06	-0.25	0.013	-0.18	0.07	-0.30	0.003
Waist-hip-ratio	0.36	0.0003	-0.39	0.0001	-0.27	0.007	-0.48	<0.001

r= Spearman correlation coefficient; P = p value; MFI, mean fluorescence intensity; BMI, body mass index.

CAPÍTULO II

Macrophage migration inhibitory factor (MIF) promoter polymorphisms (-794 CATT$_{5-8}$ and -173 G>C): relationship with mRNA expression and soluble MIF levels in young obese subjects

Research Article

Macrophage Migration Inhibitory Factor Promoter Polymorphisms (−794 CATT$_{5-8}$ and −173 G>C): Relationship with mRNA Expression and Soluble MIF Levels in Young Obese Subjects

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We analyzed the relationship of −794 CATT$_{5-8}$ and −173 G>C *MIF* polymorphisms with mRNA and soluble MIF in young obese subjects. A total of 250 young subjects, 150 normal-weight and 100 obese subjects, were recruited in the study. Genotyping of −794 CATT$_{5-8}$ and −173 G>C *MIF* polymorphisms was performed by PCR and PCR-RFLP, respectively. MIF mRNA expression was determined by real-time PCR and serum MIF levels were measured using an ELISA kit. For both *MIF* promoter polymorphisms, no significant differences in the genotype and allele frequencies between groups were observed. MIF mRNA expression was slightly higher in obese subjects than in normal-weight subjects (1.38-fold), while soluble MIF levels did not show differences between groups. In addition, we found an increase in MIF mRNA expression in carriers of the 6,6 and C/C genotypes and the 6G haplotype of the −794 CATT$_{5-8}$ and −173 G>C *MIF* polymorphisms, although it was not significant. In conclusion, this study found no relationship between obesity and *MIF* gene promoter polymorphisms with MIF mRNA expression in young obese subjects.

1. Introduction

Obesity is a chronic, complex, and multifactorial disease characterized by a state of chronic low-grade systemic inflammation. This chronic inflammation is involved in insulin resistance (IR), which is the underlying condition of type 2 diabetes mellitus (T2DM) and metabolic syndrome [1, 2]. Several studies have shown that obesity is associated with elevated serum levels of a wide range of inflammatory markers including C-reactive protein (CRP), interleukin 6 (IL-6), interleukin 8 (IL-8), and monocyte chemoattractant protein 1 (MCP-1) [3, 4].

Macrophage migration inhibitory factor (MIF) is a protein with a molecular weight of 12.5 kDa [5]; it was one of the first cytokines reported in 1966 and described as a T cell derived cytokine that inhibited the random migration of macrophages *in vitro* and promoted macrophage accumulation during delayed-type hypersensitivity reactions [6, 7]. Since MIF is recognized as a proinflammatory cytokine and obesity is associated with a chronic inflammatory response, MIF may have an impact on the pathophysiology of obesity [5, 8]. MIF is produced by different cells and tissues, including T cells, macrophages, monocytes, pituitary gland, fibroblasts, endothelial cells, and adipocytes [9–11]. In addition, MIF

counterregulates the immunosuppressive actions of glucocorticoids and promotes the expression and secretion of proinflammatory mediators such as tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), interleukin 2 (IL-2), IL-6, IL-8, and interferon gamma (IFNγ) [5, 12, 13].

Previous studies have reported that circulating MIF levels are elevated in rheumatoid arthritis (RA), systemic lupus erythematous (SLE), insulin resistance (IR), and type 2 diabetes mellitus (T2DM). Since these diseases are accompanied by persistent inflammation of varying degrees [14–17], it is important to conduct studies to try to elucidate the role of MIF in disease development.

An increase in soluble MIF levels has also been reported in obese subjects; several epidemiological studies relate circulating MIF levels with increased markers of inflammation and markers of beta-cell dysfunction. Furthermore, it has been observed that physical activity and a dietary-focused weight management program resulted in reduction of MIF levels in obese subjects [18–20].

The *MIF* gene is located on chromosome 22q11.23 and it has been linked with abdominal obesity in Caucasians in a genome-wide linkage scan. This may suggest that this chromosomal region is a susceptibility *locus* for abdominal adiposity in a particular population [21]. Two polymorphisms have been identified in the promoter region relative to the site of transcription with functional importance: (1) the short tandem repeat (STR) −794 CATT$_{5–8}$ MIF (rs5844572), which is a microsatellite repetition of Cytosine-Adenine-Thymine-Thymine (CATT) at position −794 bp, and the repeat length (5 to 8 repetitions) which correlates with increased gene expression and with circulating MIF levels; (2) the single nucleotide polymorphism (SNP) −173 G>C MIF (rs755622) at position −173 of the *MIF* gene with a change from Guanine (G) by Cytosine (C). The −173*C allele has been associated with mRNA expression and circulating MIF levels [22–24]. In previous reports, both functional MIF polymorphisms have been related with autoimmune/inflammatory pathologies such as RA, SLE, and psoriatic arthritis, as well as obesity and diabetes [15, 22, 25–31].

The aim of this study was to investigate the relationship of −794 CATT$_{5–8}$ and −173 G>C *MIF* polymorphisms with MIF mRNA and soluble MIF expression in young obese subjects.

2. Materials and Methods

2.1. Subjects. We recruited a total of 250 subjects, 18 to 30 years old, 150 normal-weight subjects and 100 obese subjects from the state of Guerrero, Mexico. Exclusion criteria included acute inflammatory diseases or any medication intake at the time of the investigation. All subjects gave their written informed consent prior to the study. This protocol was approved by the Research Ethics Committee of the University of Guerrero (registration number 012/2013).

2.2. Anthropometric and Clinical Measurements. Body weight was determined in light clothes and without shoes, using a Tanita body composition monitor (Tanita TBF-300 GS), and the height was measured to the nearest 0.1 cm using a stadiometer (Seca, Hamburg, Germany). From these measurements, BMI was calculated (BMI = weight/height^2, kg/m^2). Subjects were classified by BMI, obese ≥ 30 kg/m^2 and normal-weight < 24.9 kg/m^2, and by obesity class based on the criteria by the World Health Organization [32]. The body circumferences were measured with an anthropometric tape accurately within ± 0.1 cm (Seca, 201, Hamburg, Germany). Blood pressure was measured in the sitting position with an automatic sphygmomanometer (OMRON) on the left arm after 10 min rest. The systolic blood pressure (SBP) and diastolic blood pressure (DBP) were calculated from two readings with a minimal interval of 10 min.

2.3. Laboratory Measurements. A venous blood sample of 5 mL was obtained from each subject after at least 12-hour fasting. Biochemical parameters, such as total cholesterol, HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), triglycerides (TG), and fasting glucose levels, were analyzed immediately by enzymatic colorimetric methods with commercially available kits (Spinreact). The determination of MIF serum levels was performed by a commercial kit (LEGEND MAX Human Active MIF ELISA Kit, BioLegend) according to manufacturer's instructions. The MIF assay sensitivity was 17.4 ± 9.2 pg/mL. The criterion for the diagnosis of metabolic syndrome was based on the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) [33].

2.4. Genotyping of −794 CATT$_{5–8}$ and −173 G>C MIF Polymorphisms. Genomic DNA was extracted from peripheral blood leukocytes and stored at −20°C until analysis. The −794 CATT$_{5–8}$ *MIF* polymorphism was analyzed by conventional polymerase chain reaction (PCR) in a Thermal Cycler (Techne TC-412) using the following primers: 5′-TGT CCT CTT CCT GCT ATG TC-3′ (Forward) and 5′-CAC TAA TGG TAA ACT CGG GG-3′ (Reverse). Cycling conditions were as follows: initial denaturing at 94°C for 5 min followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C and then a final extension of 5 min at 72°C. Amplification products were visualized after electrophoresis on an 8% polyacrylamide gel stained with 2% AgNO$_3$. Fragments of 129, 133, 137, and 141 bp represented the −794 CATT$_5$, −794 CATT$_6$, −794 CATT$_7$, and −794 CATT$_8$ alleles, respectively.

The −173 G>C *MIF* polymorphism was genotyped by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). Amplification of a 366 bp fragment was completed using the previously reported primers [34]; 28 cycles and an annealing temperature of 60°C were used. The obtained fragment was digested with *Alu I* restriction endonuclease (New England Biolabs, Ipswich, MA, USA) by overnight incubation at 37°C. Finally, the digestion was resolved on a 6% polyacrylamide gel stained with 2% AgNO$_3$. The −173G allele resulted in 268 and 98 bp fragments while the −173C allele was identified by 206, 98, and 62 bp fragments.

2.5. MIF Expression Analysis. Peripheral blood was collected in EDTA blood collection tubes (BD Vaccutainer, NJ, USA). Immediately after blood drawing (<1 h), total leucocytes were

isolated using dextran reagent (USB Corporation, Cleveland, OH, USA), and the total RNA was obtained using Trizol reagent (Life Technologies) according to the Chomiczyki and Sacchi method [35]. The RNA concentration and purity were determined by spectrophotometry (NanoDrop 2000C, Thermo Scientific). The cDNA was synthesized from $1\,\mu$g of total RNA, using oligodT and reverse transcription reagents as indicated by the manufacturer (Promega Corporation, USA). The cDNA samples were stored at $-80°$C until the real-time PCR assays. The MIF and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was quantified using TaqMan probes and all samples were run in triplicate using the conditions indicated in the TaqMan Gene Expression Assay protocol in a Light Cycler Nano System (Roche Applied Science). Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (expressed as relative expression units), after validating similar reaction efficiencies of the interest gene (MIF) and the reference gene GAPDH by running serial dilutions of both genes [36].

2.6. Statistical Analysis. Data analysis was performed using STATA software (v.11.0) and GraphPad Prism 5 software. Differences in characteristics between groups were analyzed using the chi-square test for categorical variables (data presented as percentages), Student's *t*-test for parametric variables (data presented as mean ± SD), and Mann-Whitney *U*-test for nonparametric variables (data presented as median and 5th to 95th percentiles). The Hardy-Weinberg equilibrium test and genotype and allele frequencies were calculated by the chi-square test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Anthropometric and Biochemical Characteristics. As expected, obese subjects had higher body weight, BMI, waist circumference, hip circumference, and waist-hip ratio, as well as glucose, total cholesterol, triglycerides, and LDL-C levels than normal-weight subjects ($P < 0.05$). There were no significant differences in age, gender, or HDL-C levels between groups ($P > 0.05$) (data not shown).

Anthropometric and biochemical characteristics as well as metabolic abnormalities of study subjects according to gender are shown in Table 1. In both groups of normal-weight and obese subjects, body weight, height, waist circumference, waist-hip ratio, and systolic blood pressure parameters were higher in men than in women ($P < 0.05$), whereas men with normal weight had low HDL-C levels and body fat mass ($P < 0.05$) and obese men had high TG levels ($P = 0.008$) compared with the women from each respective group.

Table 1 also shows the prevalence of metabolic syndrome and its components, where we found in the normal-weight group higher prevalence of hypertension (12% versus 2%, $P = 0.010$) and hypercholesterolemia (20% versus 6%, $P = 0.013$) in men than in women. In the obese group, the prevalence of hypertension (41% versus 16%, $P = 0.005$), impaired fasting glucose (8% versus 0%, $P = 0.04$), hypertriglyceridemia (51% versus 25%, $P = 0.009$), and metabolic syndrome was higher in men than in women (49% versus 28%, $P = 0.032$).

3.2. Distribution of -794 CATT$_{5-8}$ and -173 G>C MIF Polymorphisms. Both *MIF* promoter polymorphisms analyzed were in Hardy-Weinberg equilibrium in the control group (-794 CATT$_{5-8}$, $P = 0.88$ and -173 G>C, $P = 0.44$). The distributions of -794 CATT$_{5-8}$ and -173 G>C *MIF* polymorphisms in normal-weight and obese subjects are shown in Table 2. The comparative analysis of genotype and allele frequencies of -794 CATT$_{5-8}$ and -173 G>C *MIF* polymorphisms between groups did not show significant differences. We also compared the clinical and biochemical variables by genotypes of both *MIF* polymorphisms, but we did not observe significant differences (data not shown). Additionally, we performed haplotype analyses of both polymorphisms considering the following combinations: 5G, 6G, and 7C. The estimated frequencies of the 5G, 6G, and 7C haplotypes were 14%, 48%, and 38%, respectively, in the total population (data not shown).

3.3. Relationship of MIF Promoter Polymorphisms with Its Expression in the Studied Groups. Relative MIF mRNA expression in total leucocytes was slightly higher in obese subjects than in normal-weight subjects (1.38-fold) (Figure 1). To investigate the functional impact of both polymorphisms, the quantitative MIF mRNA expression among the different genotypes for both polymorphisms was analyzed. When we analyzed the expression according to the STR -794 CATT$_{5-8}$ *MIF*, we found that carriers of the 6,6 genotype had slightly higher expression in comparison to the 7,7 genotype, and the latter with respect to the 5,5 genotype, in the total population (1.38 > 1.08 > 1) (Figure 2(a)). Similarly, when we compared the expression by groups, a modest increase of MIF mRNA expression was observed in the 6,6 carriers in both groups, while the 7,7 carriers had a low expression in the obese group. Additionally, the 6,6 obese carriers expressed slightly higher mRNA expression than normal-weight 6,6 carriers (Figure 2(b)). Carriers of -173 C/C genotype had a slightly higher expression than carriers of the G/G genotype in the total population (1.47 > 1) (Figure 3(a)). When we compared the expression by groups, a modest increase of MIF mRNA expression was observed in the carriers of the C/C genotype compared to the G/G genotype in both groups (Figure 3(b)). To analyze the combined effect of -794 CATT$_{5-8}$ and -173 G>C *MIF* polymorphisms on the MIF mRNA expression, we analyzed the expression according to 5G, 6G, and 7C haplotypes. We found that the carriers of the 6G haplotype had the highest expression in comparison to the 7C haplotype, and the latter with respect to the 5G haplotype in the total population (1.38 > 1.21 > 1), although it was not significant (Figure 4(a)). Similarly, in both groups, the carriers of the 6G haplotype had high MIF mRNA expression (Figure 4(b)).

3.4. Serum MIF Levels and MIF Promoter Polymorphisms. We analyzed MIF serum levels in obese and normal-weight subjects, but we did not find significant differences between both groups ($P = 0.44$) (Figure 5). When MIF serum levels were analyzed according to the -794 CATT$_{5-8}$ and -173 G>C *MIF* polymorphisms, we did not observe significant differences (data not shown). Furthermore, a correlation

TABLE 1: Clinical and biochemical characteristics by study group.

Variables	Normal-weight ($n = 150$)			Obese ($n = 100$)		
	Male ($n = 56$)	Female ($n = 94$)	P value	Male ($n = 49$)	Female ($n = 51$)	P value
Age (years)[c]	21 (18–25)	20 (18–26)	0.74	22 (19–28)	21 (18–25)	0.18
Weight (kg)[c]	62 (51.4–76.3)	52.4 (43.4–65.3)	<0.001	99.7 (82.7–126.7)	84.8 (74.3–108)	<0.001
Height (cm)[c]	169.5 (157–183)	156.5 (148.5–166)	<0.001	171 (160–182)	160 (151–171)	<0.001
BMI (kg/m^2)[c]	21.7 (19.1–24.6)	22.2 (18.7–24.5)	0.86	33.9 (30–43.1)	33.8 (30–40.4)	0.90
Obesity[a]						0.88
Class I (30–34.9 kg/m^2)	—	—	—	32 (65)	33 (65)	
Class II (35–39.9 kg/m^2)	—	—	—	13 (27)	15 (29)	
Class III (≥40 kg/m^2)	—	—	—	4 (8)	3 (6)	
Waist circumference (cm)[c]	79 (70–89)	75.2 (65–88)	0.0001	109 (99–131)	103 (89–116)	0.0002
Hip circumference (cm)[c]	94 (86–104)	93.4 (87–103)	0.48	115 (107–129)	118 (109–131)	0.07
Waist-hip ratio[b]	0.84 ± 0.05	0.80 ± 0.06	0.0003	0.94 ± 0.05	0.87 ± 0.07	<0.001
Body fat mass (%)	13.4 (8.2–20.5)	24 (15–32.4)	<0.001	33.9 (25.6–44.1)	42.1 (34.9–49.5)	<0.001
Body fat mass (kg)	8.5 (4.4–14.8)	12.4 (6.7–21)	<0.001	33.1 (23.9–55.2)	35.4 (25.9–52.7)	0.064
SBP (mmHg)[c]	115 (94–140)	103.5 (88–121)	<0.001	125 (103–141)	114 (98–134)	<0.001
DBP (mmHg)[b]	68.5 ± 8.3	68.3 ± 7.9	0.87	76.9 ± 10.7	73.8 ± 8.5	0.11
Metabolic profile						
Glucose (mg/dL)[c]	85.5 (70–104)	83 (68–98)	0.30	89 (76–114)	88 (71–103)	0.52
Cholesterol (mg/dL)[c]	156 (120–244)	152 (101–212)	0.11	167 (110–234)	171 (113–227)	0.43
Triglycerides (mg/dL)[c]	80.5 (40–188)	70 (42–167)	0.12	150 (63–420)	118 (51–287)	0.008
LDL-C (mg/dL)[c]	87.5 (33–207)	88 (37–158)	0.80	120 (72–184)	102 (50–187)	0.09
HDL-C (mg/dL)[c]	38 (26.5–58.8)	42 (25.3–65)	0.035	39 (28–60)	42 (31–63)	0.55
Metabolic syndrome						
Hypertension (≥130/85 mmHg)[a]			0.010			0.005
No	49 (88)	92 (98)		29 (59)	43 (84)	
Yes	7 (12)	2 (2)		20 (41)	8 (16)	
Glucose (>110 mg/dL)[a]			0.19			0.04
No	55 (98)	94 (100)		45 (92)	51 (100)	
Yes	1 (2)	0 (0)		4 (8)	0 (0)	
Hypercholesterolemia (≥200 mg/dL)[a]			0.013			0.67
No	45 (80)	88 (94)		41 (84)	41 (80)	
Yes	11 (20)	6 (6)		8 (16)	10 (20)	
Hypertriglyceridemia (≥150 mg/dL)[a]			0.050			0.009
No	45 (80)	85 (91)		24 (49)	38 (75)	
Yes	11 (20)	8 (9)		25 (51)	13 (25)	
Metabolic syndrome[a]			0.70			0.032
No	55 (98)	93 (99)		25 (51)	36 (72)	
Yes	1 (2)	1 (1)		24 (49)	14 (28)	

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, LDL-C: low density lipoprotein-cholesterol, and HDL-C: high density lipoprotein-cholesterol. [a]Data presented as n and percentage. Chi-square test. [b]Data presented as mean ± SD. Student's t-test. [c]Data presented as median and 5th and 95th percentile. Mann-Whitney U-test.

analysis between MIF serum levels and measures of central adiposity was performed. In both groups of normal-weight and obese subjects, we did not observe a positive correlation between MIF serum levels with body weight, BMI, waist and hip circumferences, and waist-hip ratio ($P > 0.05$) (data not shown).

4. Discussion

This study shows that the MIF mRNA expression in total leucocytes is slightly increased in obese subjects when compared with the normal-weight group. However, we did not find a significant association between −794 CATT$_{5-8}$ and −173 G>C

TABLE 2: Genotype and allele frequencies of −794 CATT$_{5-8}$ (rs5844572) and −173 G>C (rs755622) *MIF* polymorphisms in normal-weight and obese subjects.

Polymorphism	Normal-weight $n = 150$ (%)	Obese $n = 100$ (%)	P^* value
rs5844572			
Genotype			0.53
5,5	4 (3)	2 (2)	
5,6	20 (13)	18 (18)	
5,7	22 (15)	18 (18)	
6,6	48 (32)	22 (22)	
6,7	44 (29)	29 (29)	
7,7	12 (8)	11 (11)	
Allele			0.23
5	50 (17)	40 (20)	
6	160 (53)	91 (46)	
7	90 (30)	69 (35)	
rs755622			
Genotype			0.15
GG	68 (45)	37 (37)	
GC	69 (47)	47 (47)	
CC	13 (8)	16 (16)	
Allele			0.07
G	205 (68)	121 (61)	
C	95 (32)	79 (39)	

*Chi-square test χ^2.

MIF polymorphisms with MIF mRNA expression in young obese subjects.

The 6,6 genotype frequency for −794 CATT$_{5-8}$ polymorphism and the frequency of the G allele for −173 G>C polymorphism were similar to previous studies in Mexican Mestizos from western Mexico populations [15, 27, 28, 37]. Conversely, for −794 CATT$_{5-8}$ *MIF* polymorphism, the 5,6 genotype was the most frequent in a Japanese population [29] and, for the SNP −173 G>C, the C allele was the most frequent in Caucasian patients with psoriatic arthritis [38]. In the case of the −794 CATT$_{5-8}$ *MIF* polymorphism, we did not observe the presence of genotypes with the −794 CATT$_8$ high-expression allele which was reported as low frequency (1%) in Mexican Mestizo patients with RA and 0.4% in Japanese subjects [27, 29]. These differences may be attributed to the sample size and the inclusion criteria in each study, as well as to the racial influence among populations with different ethnic origin, thus conferring a greater genetic diversity in the distribution of these and other polymorphisms [39, 40].

Several studies have reported the relative MIF mRNA expression in obese subjects. Dandona and coworkers showed that MIF mRNA expression in mononuclear cells is significantly increased in obese patients compared to the control group and is related with plasma free fatty acids (FFA) concentrations and BMI but not MIF plasma concentrations or HOMA-index [18]. In another study, increased
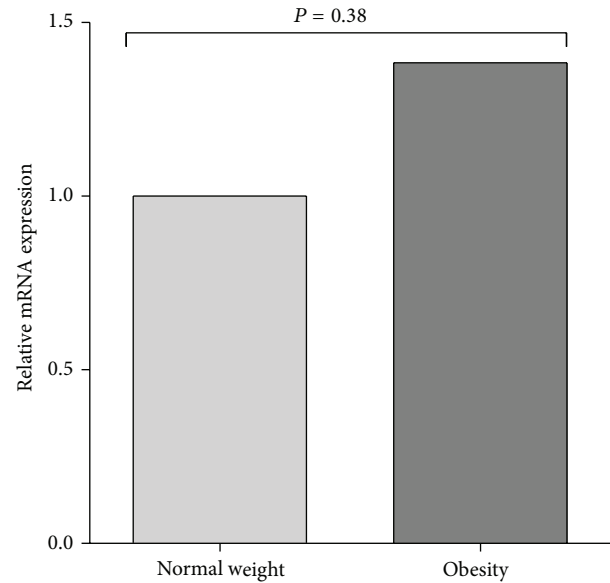

FIGURE 1: Relative MIF mRNA expression in normal-weight and obese subjects. Note that the obese subjects had a modest increase in MIF mRNA expression when compared with normal-weight subjects. Relative expression analysis was performed using the $2^{-\Delta\Delta Ct}$ method and *GAPDH* as the reference gene. Comparison among groups was performed using Mann-Whitney U-test; $P < 0.05$.

MIF mRNA expression in mononuclear cells was observed in obese subjects and correlated with BMI [41]. Our results suggest the involvement of MIF in the pathophysiology of obesity and its relationship with metabolic comorbidities.

Very few studies have reported the relationship between *MIF* gene polymorphisms and obesity. In 2006, Sakaue and coworkers found that −794 CATT$_{5-8}$ *MIF* polymorphism was associated with obesity in a Japanese population [29]. In another study, the 6,7 genotype of the MIF −794 CATT$_{5-8}$ polymorphism was associated with susceptibility to acute coronary syndrome in a western Mexican population [37]. To our knowledge, this is the first study that reports the relationship between *MIF* gene polymorphisms and MIF mRNA expression in obese young subjects. For the −794 CATT$_{5-8}$ polymorphism, carriers of the 6,6 genotype had slightly high MIF mRNA expression in comparison to the other genotypes in obese and nonobese subjects. Besides, obese 6,6 carriers expressed high mRNA in comparison with the normal-weight 6,6 carriers, although it was not statistically significant. Previously, it has been reported that this repeat regulates basal and stimulus-induced transcriptional activity, which increases almost proportionally with repeat number in defined *in vitro* assay systems. Reporter gene assays have demonstrated that the CATT$_5$ allele has the lowest basal level and stimulated MIF promoter activity compared to the CATT$_6$ and CATT$_7$ allele *in vitro* [22, 42], but it is unknown which type of transcription factor regulates MIF expression through the promoter region containing the CATT repeat, while carriers of the CATT$_7$ allele show high circulating MIF levels [24].
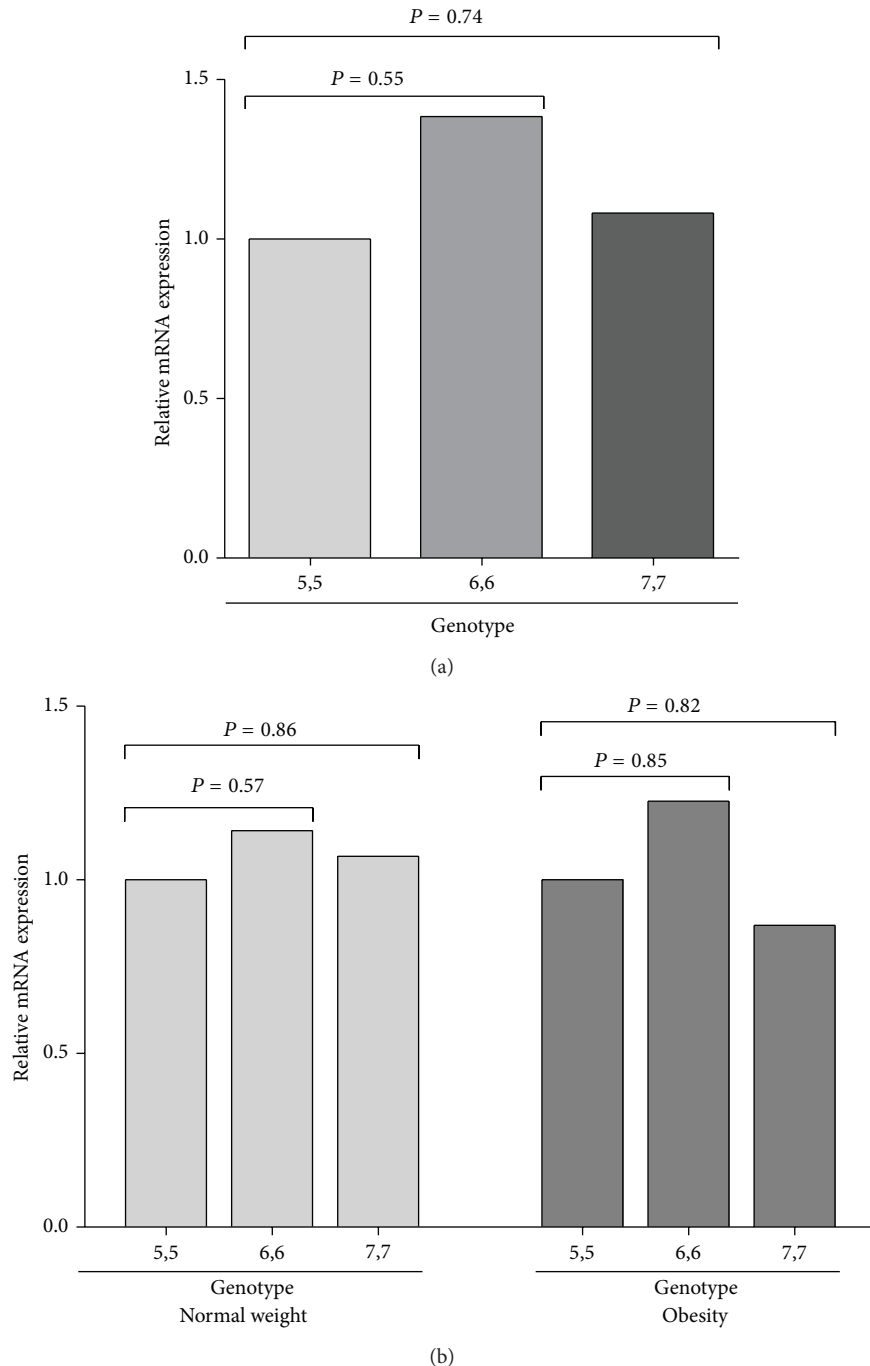
(a)

(b)

FIGURE 2: Relative MIF mRNA expression by -794 $CATT_{5-8}$ *MIF* (rs5844572) genotypes in normal-weight and obese subjects. (a) The slightly high MIF mRNA expression was observed in the 6,6 carriers, while the 5,5 carriers had lower expression in the total population. (b) The modest increase in MIF mRNA expression was observed in the 6,6 carriers in both groups, while the 7,7 carriers had lower expression in the obese group. Relative expression analysis was performed using the $2^{-\Delta\Delta Ct}$ method, using *GAPDH* as the reference gene. Comparison among groups was performed using Mann-Whitney U-test; $P < 0.05$.

For the -173 G>C polymorphism, we found that the carriers of the C/C genotype had slightly high *MIF* mRNA expression in comparison to the G/G genotype, in obese and nonobese subjects, although it was not statistically significant. In reporter gene analyses, it has been shown that the -173 G>C polymorphism plays a role in the gene transcriptional regulation in a cell-type dependent manner in which the C allele promotes transcription in a human T lymphoblast cell line (CEMC7A), while the G allele favors transcription in a lung epithelial cell line (A549). These changes in expression could be due to differences in the transcription factor interaction with the MIF -173 element.
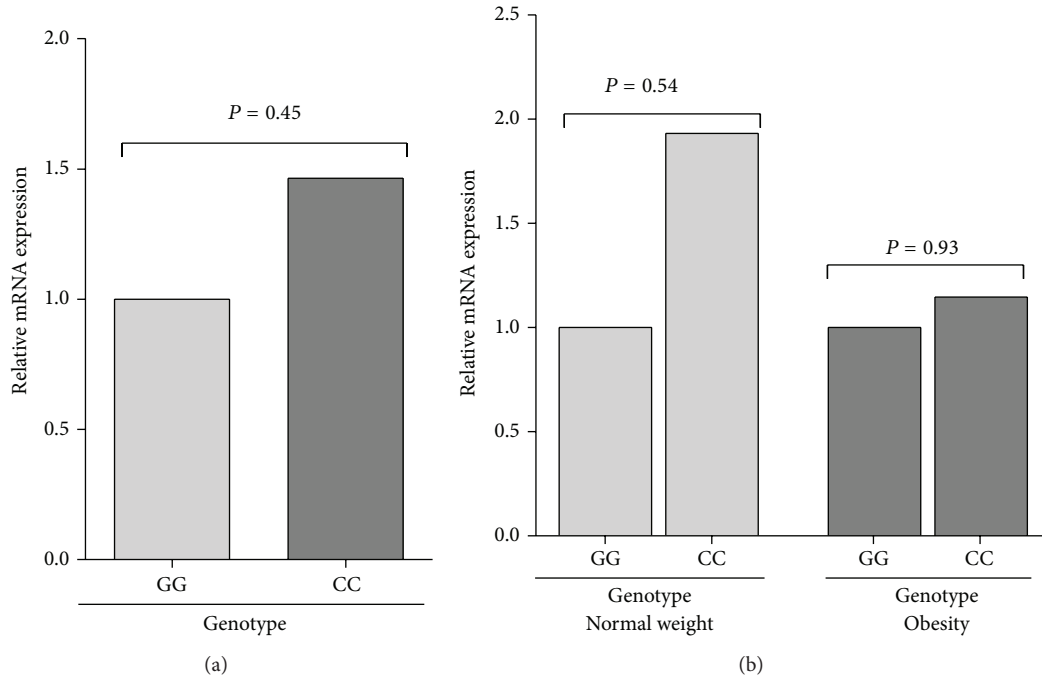
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FIGURE 3: Relative MIF mRNA expression by −173 G>C *MIF* (rs755622) genotypes in normal-weight and obese subjects. (a) The slightly high MIF mRNA expression was observed in the CC carriers in the total population; (b) the GG carriers had lowest expression in both groups. Relative expression analysis was performed using the $2^{-\Delta\Delta Ct}$ method, using *GAPDH* as the reference gene. Comparison among groups was performed using Mann-Whitney U-test; $P < 0.05$.

Based on the promoter sequence analysis, AP-4 transcription factor is a particular candidate [23]. Furthermore, the C allele is associated with increased circulating MIF levels [23, 24]. These findings provide a biological support to the results of the present study.

As mentioned above, the two polymorphisms have genetic effects on promoter activity through interactions *in vitro*; therefore the functional impact of the polymorphism should be considered with respect to the haplotype. We found that the carriers of the 6G and 7C haplotypes had a modest increase in MIF mRNA expression in comparison to the 5G haplotype in the total population, but in obese subjects and controls the carriers of the 6G haplotype had a tendency to increase MIF mRNA expression. Allele 6 was found more frequently in our population and in other studies it has been identified as a high-expression allele together with alleles 7 and 8; therefore the increase in MIF expression can be attributed to allele 6. This finding is consistent with a previous study in a reporter gene assay, where it was shown that the 6G haplotype had the highest MIF promoter activity in the A549 epithelial cell line, suggesting functional importance of the MIF promoter haplotype in determining levels of MIF gene transcription [43]. Furthermore, in Caucasian and African American populations with SLE, the 7C haplotype is associated with high circulating MIF levels [26]. In addition, the −794 CATT$_7$ and −173C polymorphisms were in linkage disequilibrium in a Mexican Mestizo population ($D' = 0.87$, $P < 0.001$), which indicates that both alleles are segregated

in block from one generation to another and may confer a similar risk [27].

Also, we did not find significant differences between MIF serum levels in both groups. However, others studies have shown increased MIF serum levels in subjects with obesity and type 2 diabetes. Dandona et al. reported a correlation between serum MIF levels and the body mass index (BMI), finding that obese subjects with an average BMI of 37.5 ± 4.9 kg/m^2 had a significant higher fasting MIF concentration (2.8 ± 2.0 ng/mL) than lean control subjects (BMI 22.6 ± 3.4 kg/m^2; 1.2 ± 0.6 ng/mL) [18]. Similarly, increased MIF serum levels were found in overweight adolescents compared with those of normal weight, and MIF concentrations were associated with markers of inflammation and obesity [20]. Other studies confirmed elevated MIF plasma levels in obese individuals compared to lean subjects [41, 44]. Also, the effect of some medications and the reduction of body weight on circulating MIF levels have been determined. In obese subjects with metformin treatment, an antidiabetes drug decreased MIF plasma concentrations of 2.3 ± 1.4 to 1.6 ± 1.2 ng/mL after an intervention of 6 weeks, and, after withdrawal of the drug, MIF levels returned to their initial value indicating a metformin-dependent effect [18]. In addition, morbidly obese subjects who participated in diet and physical activity based weight management programs showed a significant decrease in circulating MIF concentrations after weight loss of 14.4 kg [19]. In another weight loss program, weight reduction of 4.4 kg was achieved with a 67% decrease in
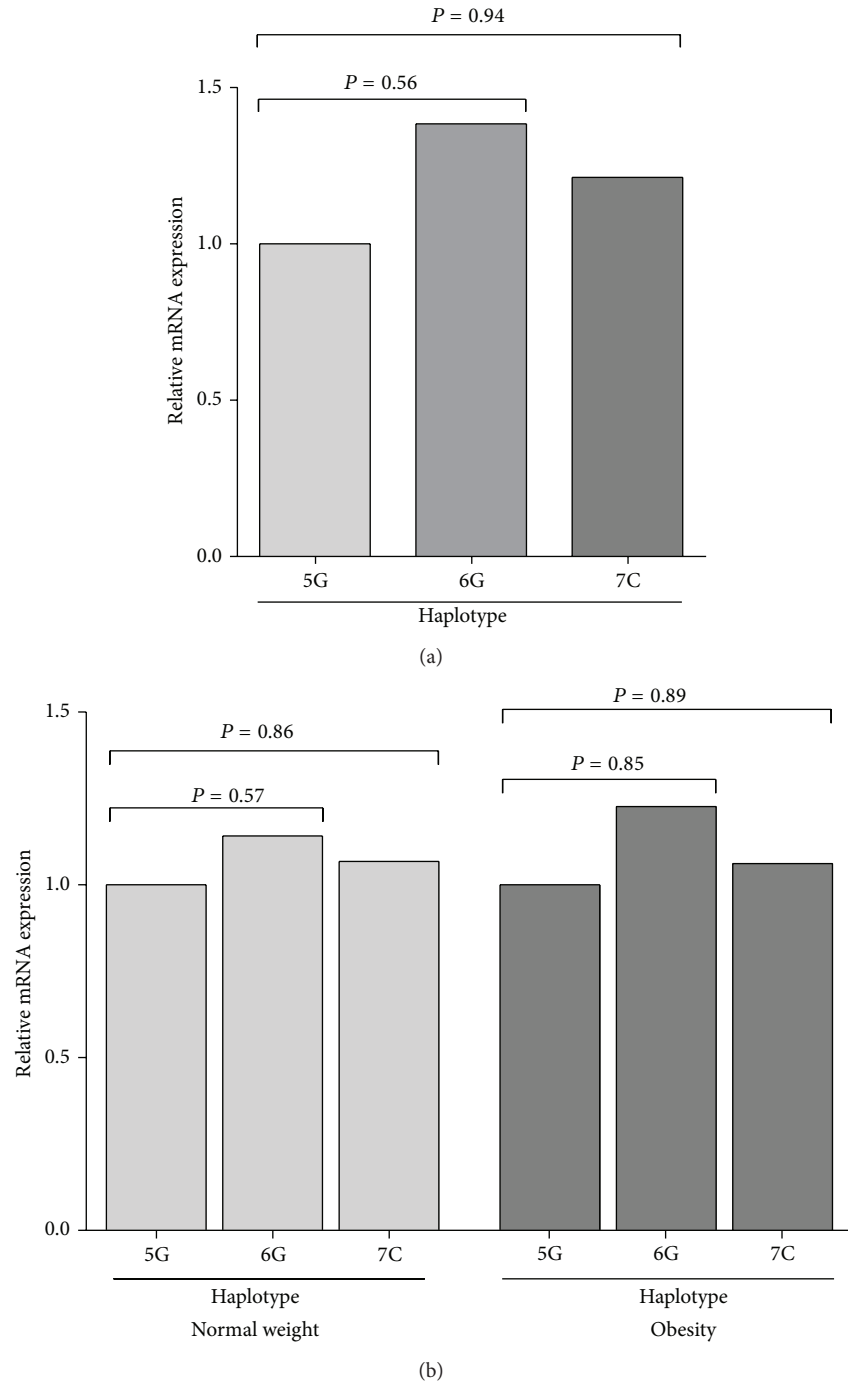
(a)

(b)

FIGURE 4: Relative MIF mRNA expression by -794 CATT$_{5-8}$ (rs5844572) and -173 G>C *MIF* (rs755622) haplotype in normal-weight and obese subjects. (a) The slightly higher MIF mRNA expression was observed in the 6G carriers, while the 5G carriers had a low expression in the total population. (b) The modest increase in MIF mRNA expression was observed in the 6G carriers in both groups. Relative expression analysis was performed using the $2^{-\Delta\Delta Ct}$ method, using *GAPDH* as the reference gene. Comparison among groups was performed using Mann-Whitney U-test; $P < 0.05$.

circulating levels of MIF [45]. In contrast to these studies, however, morbid obese subjects with BMI of 46.7 ± 5.8 kg/m^2 show low plasma MIF levels (about 0.2 ± 0.4 ng/mL); after gastric restrictive surgery, the BMI decreased markedly (33 ± 4.8 kg/m^2) while MIF concentrations remained low for 6

months during weight loss, after which they significantly increased to normal levels at 24 months postoperatively [46]. The relationship between obesity and MIF is not consistent and any causal relationship between obesity and MIF levels remains to be established [47]. Factors that may contribute to
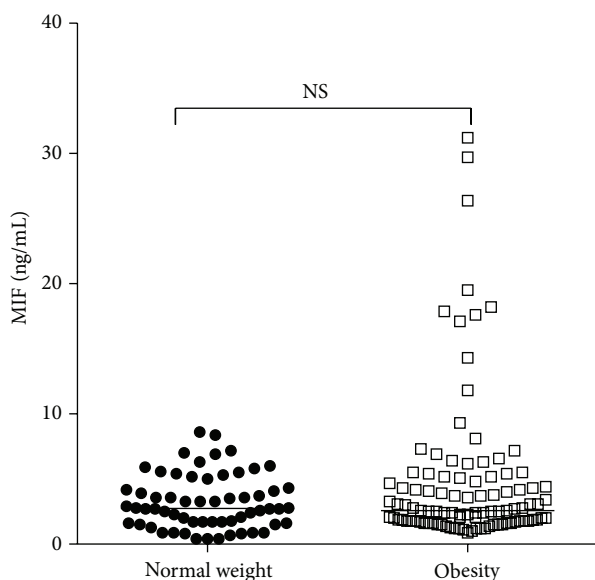
FIGURE 5: MIF serum levels by study groups. Data expressed as median and percentiles (p5–p95). Mann-Whitney U-test. NS: nonsignificant.

the variability in these studies include differences in gender, since MIF plasma levels are higher in males [30], the use of hormone replacement therapy (HRT), since women with HRT show 2-3-fold higher plasma MIF levels [19], circadian rhythm [48], and differences in *MIF* promoter genotypes leading to variations in promoter activity and MIF serum levels [22–24, 30]. However, the −794 CATT$_{5-8}$ and −173 G>C *MIF* polymorphisms did not show significant differences with MIF serum levels in our study, results similar to those reported in Mexican Mestizo patients with RA [27], SLE [15], and psoriatic arthritis [28]; however, they were not able to replicate the association of *MIF* polymorphisms with MIF serum levels; this could be due to differences in the genetic structure of our population which may influence activity at the *MIF* gene locus.

Our results showed no correlation between mRNA expression and MIF serum levels, where the obese subjects had a slightly higher mRNA expression but not MIF serum levels in comparison with the normal-weight group. It is known that the mRNA expression of a particular gene is not always predictive of protein expression, and the correlation between the two can vary significantly [49]. There are several possible explanations for the differences between the mRNA and protein levels and these may not be mutually exclusive, including complex posttranscriptional mechanisms and variation in protein half-lives because cells can control the protein level in the cell through the rates of degradation or synthesis for a given protein, as well as by the different sensitivities in methodologies for detecting mRNA and protein expression [50]. These possibilities could explain our data. To understand the reasons for this discordance, the dynamic processes involved in synthesis and degradation of MIF must be investigated in future studies.

Finally, some limitations of our study should be considered such as the heterogeneity of comorbidities of our study subjects and, in reference to our small sample size, a greater obese group is desirable to improve the power of the study.

In summary, we did not find the evidence to support the relationship between obesity and *MIF* gene promoter polymorphisms with MIF mRNA expression in young obese subjects.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.
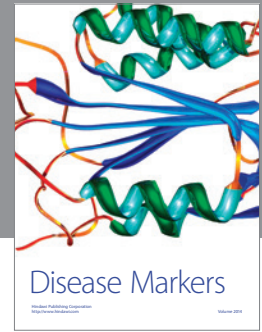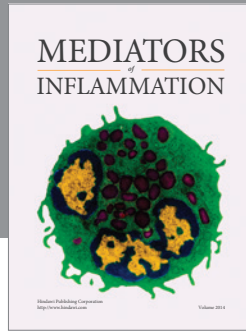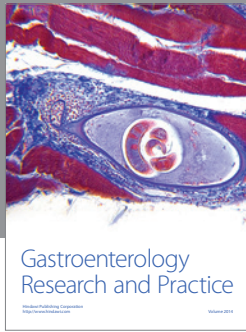
Acknowledgments

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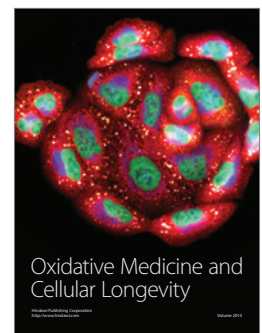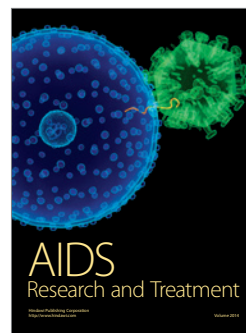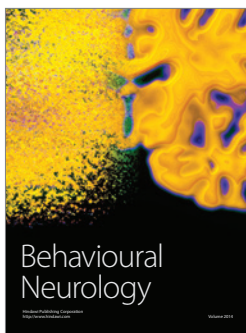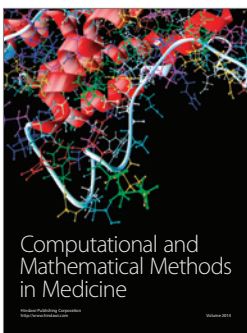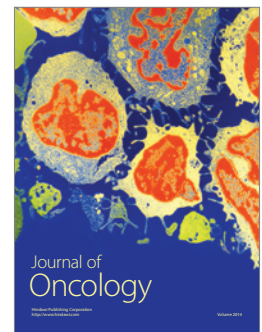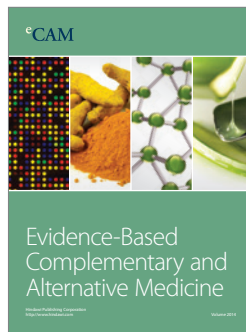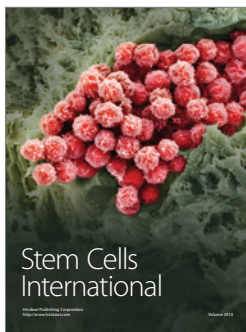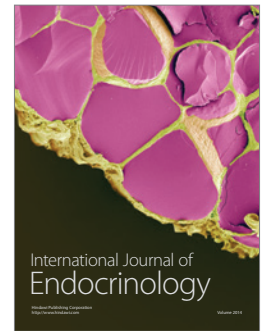
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Capitulo III

Correlation between cytokine profile and metabolic abnormalities in young subjects

Correlation between cytokine profile and metabolic abnormalities in young subjects

Abstract

Introduction: Chronic systemic inflammation characterized by elevated circulating cytokines and chemokines, is an important feature of obesity. This study assessed the relationship between cytokines and high sensitivity C-reactive protein with metabolic alterations in obese young subjects.

Material and methods: A total of 100 subjects were recruited from the state of Guerrero, Mexico. All individuals had an age range of 18 to 30 years old and were divided into two groups: normal-weight (n=50) and obese subjects (n=50). The levels of circulating cytokines (IL-6, IL-1β, IFN-ɣ, IL-2, IL-12, IL-4, TNF-α, IL-13, IL-17 and IL-10) were measured using a bead based multiplex system. MIF levels were determined by ELISA. Serum hsCRP was analyzed by turbidimetry.

Results: We found increased serum concentrations of IL-6 and hsCRP in subjects with overall and abdominal obesity. Furthermore, subjects with hypertriglyceridemia had higher serum hsCRP levels compared to those subjects without dyslipidemia. In addition, the results showed a positive correlation between adiposity measures and circulating levels of IL-6 and hsCRP, but a negative correlation with IL-10 levels. No significant differences were found for serum levels of MIF, IL-1β, IFN-ɣ, IL-2, IL-12, IL-4 and IL-10 neither between both study groups nor according to metabolic abnormalities.

Conclusion: The results show that hsCRP, IL-6 and IL-10 are the main inflammatory markers related to obesity and/or dyslipidemia in young subjects. Therefore, these markers may be useful in the early detection of comorbidities in obese population.

Key words: obesity, inflammation, cytokines, high sensitivity C-reactive protein.

Introduction

Obesity is associated with chronic low-grade systemic inflammation and is one of the key factors for the development of metabolic diseases such as insulin resistance (IR), type 2 diabetes mellitus (T2DM), hypertension, hyperlipidemia, atherosclerosis, metabolic syndrome and cardiovascular disease (CAD) [1-4]. There is accumulating evidence that deregulated production of cytokines in obesity contributes to the low-grade chronic inflammation, which is recognized as an important player in the pathogenesis of obesity-associated comorbidities [5-7]. Several studies have reported increased circulating levels of a wide range of inflammatory markers including C-reactive protein (CRP), interleukin 6 (IL-6), interleukin 8 (IL-8), monocyte chemoattractant protein 1 (MCP-1) and macrophage migration inhibitory factor (MIF) in obese and T2DM individuals, and were positively correlated to BMI (body mass index) and waist circumference [8-10].

Previous studies in our population have reported the prevalence of obesity, hypertension and other cardiovascular risk factors in children and adults [11-13]. In addition to traditional risk factors for the development of T2DM and CAD such as obesity, hypertension and dyslipidemias, chronic inflammation is now recognized as

an important risk factor involved in the pathogenesis of these diseases [14-15]. Several studies have reported the relationship of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and IL-6 with insulin resistance and atherogenesis [16-17]. Furthermore, IL-6 and activin-A were recognized as major risk factors for cardiovascular events and mortality in T2DM subjects [18]. However, the role of the T-helper (Th) 1 and Th2 cytokines has not been sufficiently studied in obesity, T2DM and CAD. Previously, it was reported a mixed Th1-Th2 serum cytokine profile in subjects with metabolic syndrome (MetS) as a major risk factor for T2DM (if not present already) and CAD [19]. In another study, T2DM subjects showed a mixed Th1-Th2 profile and T2DM-CAD subjects presented enhanced Th1 polarization similar to that of CAD subjects with further reduction in their Th2 cytokine levels [20]. This study assessed the relationship between cytokine profiles and high sensitivity C-reactive protein with metabolic alterations in obese young subjects.

Material and methods

Subjects

A total of 100 subjects were recruited from the state of Guerrero, Mexico. All individuals had an age range of 18 to 30 years old, and were divided into two groups: 50 with normal-weight and 50 obese subjects. Subjects were randomly selected from the general population; exclusion criteria were acute or chronic infections, being under any medication, pregnancy and presence of autoimmune or chronic inflammatory diseases. All subjects gave written informed consent prior enrollment in the study. This protocol was approved by the Research Ethics Committee of the University of Guerrero.

Anthropometric measurements

Body weight was determined in subjects wearing light clothes and without shoes, using a body composition monitor (Tanita TBF-300 GS). The height was measured to the nearest 0.1 cm using a stadiometer (Seca, Hamburg, Germany). From these measurements, BMI was calculated (BMI = weight/height2, kg/m2). Subjects were classified by BMI: obese ≥30 kg/m^2 and normal-weight <24.9 kg/m^2, based on the criteria of World Health Organization [21]. The body circumferences were measured with an anthropometric tape accurate to within ±0.1 cm (Seca, 201, Hamburg, Germany).

Biochemical analysis

A venous blood sample of 5 mL was obtained from each subject after at least 12 hours fasting. Biochemical parameters, such as total cholesterol (TC), HDL-cholesterol (HDL-c), LDL-cholesterol (LDL-c), triglycerides (TG) and fasting glucose levels were determined in serum samples by enzymatic colorimetric methods with commercially available kits (Spinreact, Spain). Abnormal biochemical levels were identified when TC ≥200 mg/dL, TG ≥150 mg/dL, and glucose >110 mg/dL, based on the NCEP Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) [22].

Determination of serum hsCRP and cytokines levels

The levels of cytokines (IL-6, IL-1β, IFN-γ, IL-2, IL-12, IL-4, TNF-α, IL-13, IL-17 and IL-10) were measured in serum samples using the Human Cytokine Magnetic 10-plex custom kit (Invitrogen Life Technologies, USA) and the MAGPIX® System (Luminex, USA). Concentrations of TNF-α, IL-13 and IL-17 were below the detection limit of the

multiplex assay and thus were excluded from the statistical analysis. Serum samples were stored at -80°C until the day of the assay and processed according to the manufacturer's instructions. The fluorescence values of 100 events per region were considered as quantification criteria. We performed serial dilutions of the recombinant standards provided in the assay to generate standard curves of the cytokines in duplicate. Curves were adjusted to a logistic regression model (5 parameters) and showed correlation coefficients (R^2) above 0.95. Quantitative levels of cytokines in samples were interpolated from the standard curves and reported in pg/mL.

The determination of serum MIF levels was performed by enzyme-linked immunosorbent assay (LEGEND MAX^{TM} Human Active MIF ELISA Kit, BioLegend) according to manufacturer's instructions. The MIF assay sensitivity was 17.4 ± 9.2 pg/mL. High sensitivity C-reactive protein (CRP) was measured by turbidimetry in the BS-120 chemistry analyzer (MINDRAY, China), the detection limit was less than 1 mg/L.

Statistical analysis

Data analysis was performed using STATA software (v.11.0) and GraphPad Prism (v 5.0). Differences in characteristics between groups were analyzed using the chi-square test for categorical variables (data presented as percentages), Student's *t*-test for parametric variables (data presented as mean±SD) and Mann-Whitney *U*-test for nonparametric variables (data presented as median and 5th to 95th percentiles). Correlations between variables were expressed as Spearman's correlation coefficients. $P < 0.05$ was considered statistically significant.

Results

Anthropometric, biochemical and inflammatory characteristics by group are shown in Table I. As expected, obese subjects had higher body weight, BMI, waist and hip circumferences, waist-hip-ratio and body fat mass ($p<0.001$) as well as triglycerides concentrations ($p=0.002$) but no total cholesterol, HDL-c and LDL-c, in comparison to normal-weight subjects. In the comparative analysis of inflammatory markers levels by group we only found a significant increase in both IL-6 ($p=0.004$) and hsCRP concentrations ($p<0.001$) in obese subjects in comparison to normal-weight subjects. There were no significant differences for MIF, IL-1β, IFN-ɣ, IL-2, IL-12, IL-4 and IL-10 serum levels between groups.

Concentrations of serum inflammatory markers were analyzed according to metabolic abnormalities in all subjects. We found increased IL-6 ($p=0.0007$) and hsCRP serum levels ($p<0.001$), but significantly decreased IL-10 levels ($p=0.013$) in subjects with abdominal obesity when compared to those without abdominal obesity. Besides, subjects with hypertriglyceridemia had higher serum hsCRP levels ($p=0.0034$) than those subjects without dyslipidemia (Table II).

The correlation between inflammatory markers and anthropometric measures are shown in Table III. Levels of hsCRP were significantly correlated with all body measures and adiposity ($p<0.001$). Similarly, IL-6 concentrations were correlated with most measures but not with waist-hip-ratio. IL-10 levels were negatively correlated with all measures, but only significantly with hip circumference ($p=0.023$) and body mass ($p=0.03$).

In the total sample, a correlation analysis between serum cytokine concentrations was performed. We observed a positive correlation between IL-6 with IFN-ɣ (r=0.24, p=0.04) and IL-4 (r=0.25, p=0.03); IFN-ɣ with IL-2 (r=0.37, p=0.001), IL-12 (r=0.23, p=0.04), IL-4 (r=0.30, p=0.007) and IL-10 (r=0.26, p=0.023); IL-2 with IL-4 (r=0.53, p<0.001) and IL-10 (r=0.31, p=0.007); IL-12 with IL-4 (r=0.24, p=0.04) and IL-10 (r=0.29, p=0.01); and IL-4 with IL-10 (r=0.39, p=0.0005) (Table IV).

Discussion

In this study, circulating levels of hsCRP and a panel of ten cytokines and their relationship with obesity were studied in Mexican young subjects; we found increased serum levels of IL-6 and hsCRP in subjects with overall and abdominal obesity. Individuals with hypertriglyceridemia had higher serum hsCRP levels compared to those without this abnormality. Moreover, we detected a positive correlation between adiposity measures and circulating levels of IL-6 and hsCRP, but a negative correlation of these parameters with IL-10 levels.

The chronic low-grade systemic inflammation, characterized by elevated circulating cytokines and chemokines, is a prominent feature of obesity. In both children and adults, several studies have shown high circulating levels of IL-6, IL-18, MCP-1 and CRP in obese individuals [8, 9, 23-24]. Similarly, we found increased IL-6 and hsCRP serum concentrations in abdominal and overall obese subjects compared with normal-weight subjects. It is known that during obesity, IL-6 is released by the visceral adipose tissue into the portal circulation and that CRP is mainly synthesized in the liver in response to IL-6 stimulation, which would explain their proportional

increase [25]. CRP has an important effect on amplifying the inflammatory response and is used as a marker of obesity-related inflammation and as a predictor of cardiovascular events and diabetes [26, 27].

In our study, we did not find significant differences between MIF, IL-1β, IFN-ɣ, IL-2, IL-12, IL-4 or IL-10 serum levels when comparing normal-weight versus obese, nor according to metabolic abnormalities. However, previous reports on serum levels of these cytokines were inconsistent in subjects with obesity, T2DM and MetS. IL-12 levels were elevated in obesity [28, 29], MIF levels were increased in obese adolescents [30], and high circulating levels of IL-12, IFN-γ, IL-4, IL-5 and IL-13 were reported in subjects with MetS [19]. In obese adolescent girls, IL-1β, IL-4 and IL-5 levels were higher in those with central obesity than in controls [31]. Also, serum levels of IL-5, IL-10, IL-12, IL-13, IFN-γ and TNF-α were found elevated in obese subjects [32]. In other studies, inconsistent results have been reported regarding IL-10 concentrations; increased levels of this cytokine were found in obese women [33], whereas a reduction on IL-10 was reported in other study evaluating obese women, additionally, no changes were detected for this cytokine after body weight reduction in response to diet [34]. Another report detected increased IL-10 levels associated with visceral fat loss [35]. Furthermore, one of the most studied comorbidities associated with obesity is T2DM. Previously, it was reported that the presence of T2DM favors a Th1 cytokine profile in subjects with T2DM and CAD, with suppression of the Th2 cytokine profile [20]. However, it is important to mention that in our study, obese subjects do not have T2DM, only 3 obese patients had impaired fasting glucose.

In addition, the distribution of the number of metabolic abnormalities in obese subjects was as follows: 22% displayed at least one alteration, 48% exhibited two abnormalities and 30% presented three or more metabolic alterations. Thus, it is possible that obese young subjects may have an early inflammatory process where circulating levels of IL-6 and hsCRP are increased but the levels of other cytokines are difficult to be detected in peripheral blood. Also, our population of obese subjects had fewer metabolic abnormalities in comparison to former studies assessing other comorbidities. Therefore obesity alone seems to be insufficient to induce pro-inflammatory cytokine profile at an early age and the presence of other abnormalities is probably required for an increase on Th1 cytokine profile to occur. Besides, other factors that may contribute to the differences between studies are the sample size and their inclusion criteria, as well as the racial influence among populations with different ethnic origin. Despite differences in some studies, it appears to be an unregulated production of pro-inflammatory and anti-inflammatory cytokines in obesity, which probably play an important role in the pathophysiology of the disease and the development of metabolic comorbidities.

Furthermore, we detected that IL-6 and hsCRP levels were correlated with body adiposity, whereas IL-10 levels were negatively correlated with body fat mass. Similarly, other studies have reported positive correlations between IL-6 and CRP with BMI, waist and hip circumferences, and body fat percentage [8, 36, 37]. IL-6 and CRP are strongly associated because one of the main effects of IL-6 is the induction of hepatic CRP production. Therefore, both markers appear to simultaneously increase as a consequence of the inflammatory condition in obese subjects.

However, the fact that hsCRP was significantly elevated in abdominal obesity and hypertriglyceridemia, and that cytokines may drastically vary due to external influences, hsCRP may be considered a better marker of cardiometabolic risk in comparison to cytokines.

Obesity is associated with a chronic inflammatory response, abnormal adipokine production, and the activation of some pro-inflammatory signaling pathways, resulting in the induction of several biological markers of inflammation. However, the exact mechanisms have not yet been clearly elucidated. Recently, several mechanisms have been proposed as contributors to obesity-related inflammation: 1) hyperplasic and hypertrophic adipocytes synthesize pro-inflammatory adipokines such as TNF-α and IL-6; 2) macrophages migrate into the adipose tissue, where polarization from M2 to M1 macrophages is enhanced, but this polarization state depends on environmental stimuli [38]; 3) the Th2/ Th1 ratio and Treg cell activity is reduced [39]. These processes are suggested to lead to a shift in cytokine levels in obesity.

In this study, we have observed a correlation between pro- and anti-inflammatory cytokines. IFN-ɣ was correlated with IL-6, IL-12, IL-4 and IL-10; IL-4 was correlated with IL-6, IL-2, IL-12 and IL-10, and IL-10 was correlated with IL-2 and IL-12. Some previous studies on correlations between cytokines and associated comorbidities were reported in obesity. In 2006, Ranjbaran and colleagues found a correlation between pro- and anti-inflammatory cytokines in patients with coronary atherosclerosis; they demonstrated a relationship between IFN-ɣ with IL-12 and IL-10 levels [40]. IL-12 is a pro-inflammatory cytokine that induces the production of IFN-γ in T cells and natural killer cells, and promotes the differentiation of Th1 cells [41].

IFN-γ is a key mediator for IL-12 and IL-6 release by classically activated macrophages [42]. An inverse relationship was found between circulating levels of IL-10 and adiposity measures. IL-10 is a potent anti-inflammatory cytokine produced mainly by monocytes and macrophages in response to inflammatory stimulus such as IL-6 and also by regulatory T cells (T_{reg}) [43]. One may speculate that IL-10 produced by obese subjects is insufficient to decrease their inflammatory state. In fact, a recent study by Wagner et al. reported decreased circulating T_{reg} in obese individuals compared with non-obese. Moreover, the proportion of circulating T_{reg} cells was inversely correlated with indices of adiposity such as body weight and BMI, particularly in obese subjects, supporting the idea of defective anti-inflammatory pathways in obese subjects [44].

The main limitation of the present study is the small sample size. Additionally, due to the cross-sectional nature of our study we cannot determine the causal relationship between inflammatory markers and cardiometabolic abnormalities.

In conclusion, our results show that hsCRP, IL-6 and IL-10 are the main inflammatory markers related to obesity and/or hypertriglyceridemia. Therefore, these biomarkers may be a link between obesity and cardiometabolic abnormalities in young subjects.

Conflict of interest

The authors declare no conflict of interest.

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Table I. Anthropometric, biochemical and inflammatory characteristics by group.

Variable	Normal-weight (*n*=50)	Obesity (*n*=50)	*p* value
Age (years)[c]	20(18-28)	22(18-28)	0.15
Gender n(%)[a]			0.69
Male	24(48)	22(44)	
Female	26(52)	28(56)	
Weight (kg)[c]	59.7(43.1-73)	89.3(78.5-109)	<0.001
BMI (kg/m^2)[c]	22.4(18.7-24.6)	33.4(30-38.8)	<0.001
Waist circumference (cm)[c]	79.3(70.5-89)	104(90-120.5)	<0.001
Hip circumference (cm)[c]	96(87-104)	115.3(106-131)	<0.001
Waist-hip-ratio[b]	0.83± 0.05	0.9±0.07	<0.001
Body fat mass (%)[c]	17.95(9.5-32.4)	39.5(25.7-47.4)	<0.001
Body fat mass (kg)[c]	11.8(5.2-20.9)	34.4(23.9-47.9)	<0.001
Metabolic profile			
Glucose (mg/dL)[c]	84.5(73-104)	87.5(76-107)	0.05
Cholesterol (mg/dL)[b]	157±29.6	166±31	0.13
Triglycerides (mg/dL)[c]	84(42-188)	119(43-358)	0.002
LDL-c (mg/dL)[c]	109(69-207)	102(69-187)	0.42
HDL-c (mg/dL)[c]	40.5(28-68)	39(27-62)	0.68
Inflammatory markers			
MIF (ng/mL)[c]	3.3(0.9-7.2)	2.5(1.1-6.6)	0.15
IL-6 (pg/mL)[c]	1.3(0.42-7.04)	2.7(0.42-12.13)	0.004
IL-1β (pg/mL)[c]	4.5(0.6-12.01)	4.8(0.8-9.2)	0.89
IFN-ɣ (pg/mL)[c]	2.2(0.95-5.6)	2.2(0.95-4.2)	0.28
IL-2 (pg/mL)[c]	0.43(0.05-6.6)	0.23(0.05-5.5)	0.56
IL-12 (pg/mL)[c]	97(43.2-212.7)	108(56.5-255.6)	0.41
IL-4 (pg/mL)[c]	3.96(2.9-9.7)	3.96(2.9-16.8)	0.85
IL-10 (pg/mL)[c]	2.3(2.04-4.7)	2.3(1.82-3.9)	0.07
CRP (mg/L)[c]	0.57(0.16-1.78)	1.28(0.4-3.42)	<0.001

BMI, Body Mass Index; LDL-c, Low Density Lipoprotein-Cholesterol; HDL-c, High Density Lipoprotein-Cholesterol; Macrophage migration inhibitory factor, MIF; Interferon-ɣ, IFN-ɣ; Interleukin, IL. [a]Data presented as *n* and percentage. Chi-square test. [b]Data presented as mean±SD. Student *t*-test. [c]Data presented as median and 5th to 95th percentile. Mann-Whitney test.

Table II. Inflammation markers levels according to metabolic abnormalities.

Variable	MIF	IL-6	IL-1β	IFN-γ	IL-2	IL-12	IL-4	IL-10	CRP
Abdominal Obesity									
No	3.3(0.9-7.2)	1.25(0.4-7.04)	4.4(0.8-10.1)	2.2(0.95-4.2)	0.43(0.1-4.7)	95.4(46.2-194.4)	3.96(2.9-9.7)	2.3(2-3.9)	0.62(0.16-1.8)
Yes	2.5(1.1-6.4)	2.7(0.42-9.12)	4.9(0.9-8.4)	2.2(0.95-4.2)	0.14(0.1-5.5)	109(56.5-255.6)	3.96(2.9-14.4)	2.3(1.8-3.4)	1.4(0.4-3.42)
	$p=0.11$	$p=0.0007$	$p=0.60$	$p=0.86$	$p=0.60$	$p=0.31$	$p=0.76$	$p=0.013$	$p<0.001$
Glucose (>110 mg/dL)									
No	2.7(1-7.2)	1.8(0.42-7.04)	4.6(0.8-9.9)	2.2(0.95-4.2)	0.23(0.1-5.5)	107(52.1-194.4)	3.96(2.9-14.4)	2.3(1.8-3.7)	0.9(0.2-2.42)
Yes	4.4(1.5-6.4)	2.7(0.7-9.12)	4.4(0.9-8.6)	2.9(0.95-3.5)	0.1(0.1-0.43)	255.6(62.3-329.2)	3.96(3.96-3.96)	2.3(1.8-4.9)	1.8(0.28-4.64)
	$p=0.50$	$p=0.62$	$p=0.88$	$p=0.69$	$p=0.29$	$p=0.23$	$p=0.55$	$p=0.91$	$p=0.36$
Total cholesterol (≥200 mg/dL)									
No	2.9(1.1-7.2)	2.1(0.42-7.9)	4.5(0.8-9.9)	2.2(0.95-4.2)	0.23(0.1-5.5)	107(56.5-195)	3.96(2.9-14.4)	2.3(1.8-3.9)	0.9(0.22-2.41)
Yes	2.0(0.9-5.2)	1.3(0.42-4.1)	4.8(4-8.4)	2.2(1.6-4.2	0.14(0.1-2.0)	80.3(46.2-212.7)	3.96(2.9-6.2)	2.3(2-2.73)	1.04(0.15-2.5)
	$p=0.088$	$p=0.22$	$p=0.46$	$p=0.86$	$p=0.52$	$p=0.12$	$p=0.69$	$p=0.17$	$p=0.81$
Triglycerides (≥150 mg/dL)									
No	2.8(1.1-7.2)	2.1(0.42-7.9)	4.4(0.8-9.9)	2.2(0.95-4.2)	0.43(0.1-5.5)	107(53.6-212.7)	3.96(2.9-9.7)	2.3(2-3.4)	0.72(0.18-3.2)
Yes	2.7(0.9-6.4)	1.8(0.42-4.1)	4.9(0.8-9.3)	1.9(0.95-5.6)	0.23(0.1-4.2)	107(52.1-195.1)	3.96(2.9-22.9)	2.3(1.8-3.9)	1.13(0.51-2.27)
	$p=0.81$	$p=0.67$	$p=0.81$	$p=0.41$	$p=0.91$	$p=0.99$	$p=0.95$	$p=0.34$	$p=0.0034$

Data presented as median (5-95th percentile). Mann-Whitney test.

Table III. Correlation between inflammatory markers and anthropometric measures.

Variables	CRP		IL-6		IL-10	
	r	*p*	*r*	*p*	*r*	*p*
Weight	0.49	<0.001	0.26	0.02	-0.16	0.16
BMI	0.55	<0.001	0.38	0.0008	-0.22	0.05
Waist circ.	0.56	<0.001	0.34	0.003	-0.19	0.09
Hip circ.	0.51	<0.001	0.37	0.001	-0.26	0.023
Waist-hip-ratio	0.39	0.0001	0.19	0.10	-0.04	0.74
Body mass (%)	0.52	<0.001	0.38	0.0008	-0.31	0.005
Body mass (kg)	0.55	<0.001	0.36	0.002	-0.25	0.03

r = Spearman correlation coefficient; *p* = *p* value; BMI, body mass index;

waist circ., waist circumference; hip circ., hip circumference.

Table IV. Correlation between serum cytokine levels.

Cytokines	MIF		IL-6		IL-1β		IFN-γ		IL-2		IL-12		IL-4	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*P*	*r*	*p*	*r*	*p*	*r*	*p*
MIF	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL-6	0.18	0.11	-	-	-	-	-	-	-	-	-	-	-	-
IL-1β	-0.13	0.27	0.12	0.30	-	-	-	-	-	-	-	-	-	-
IFN-γ	-0.05	0.68	0.24	0.04	0.19	0.09	-	-	-	-	-	-	-	-
IL-2	-0.05	0.69	0.18	0.13	0.15	0.18	0.37	0.001	-	-	-	-	-	-
IL-12	0.025	0.83	0.20	0.08	0.0001	0.99	0.23	0.046	0.16	0.16	-	-	-	-
IL-4	0.03	0.81	0.25	0.03	0.035	0.76	0.30	0.007	0.53	<0.001	0.24	0.04	-	-
IL-10	-0.03	0.78	0.14	0.23	-0.013	0.91	0.26	0.023	0.31	0.007	0.29	0.01	0.39	0.0005

r= Spearman correlation coefficient; *p* = *p* value.

DISCUSIÓN Y CONCLUSIONES

La obesidad se asocia con una respuesta inflamatoria crónica de grado bajo, caracterizada por la producción desregulada de citocinas proinflamatorias que contribuyen a la patogénesis de resistencia a la insulina (RI), diabetes mellitus tipo 2 (DMT2), síndrome metabólico y enfermedades cardiovasculares (Hotamisligil, 2006; Fain, 2006; Lumeng *et al.,* 2011).

La inflamación de bajo grado en la obesidad se considera el producto de un sistema inmune innato activado, lo que desencadena la infiltración de células inflamatorias, tales como macrófagos, neutrófilos, células dendríticas y linfocitos, dando lugar a alteraciones cualitativas y cuantitativas de la fracción del estroma vascular del tejido adiposo blanco (Weisberg, *et al.,* 2003; Xu *et al.,* 2003; Chmelar *et al.,* 2013). La contribución del sistema inmune adaptativo a la obesidad, en particular las células T ha sido poco estudiada. Sin embargo, estudios recientes indican que la obesidad no solo se caracteriza por un aumento de la activación de células inmunes circulantes del sistema inmune innato (Poitou *et al.,* 2011), sino también del adaptativo. Viardot *et al.,* reportaron en sujetos obesos mórbidos con DMT2 que la restricción calórica con modesta pérdida de peso reduce notablemente el número de células Th1 pro-inflamatorias, con poco efecto sobre el número de células Th2 (Viardot *et al.,* 2010). Considerando lo anterior, la hipótesis que se planteó para este estudio fue que los jóvenes con obesidad presentan un número mayor de linfocitos Th1 y monocitos-macrófagos. Los principales hallazgos son: las células T $CD3^+CD4^+$, $CD3^-CD4^{int}$ y $CD3^{int}CD4^{int}$ así como las células Th1 $CD3^+CD4^+IFN\text{-}\gamma^+$, $CD3^{int}CD4^-IFN\text{-}\gamma^+$ y $CD3^{int}CD4^{int}IFN\text{-}\gamma^+$ están incrementadas en los obesos en comparación con los de peso normal, lo que puede sugerir que la inflamación sistémica reportada en obesidad incluye también células del sistema inmune adaptativo activado. Pocos estudios han abordado la relación de las células Th circulantes con obesidad. En el 2006, fue reportado el predominio de células Th1 en niños con obesidad, además el número de células Th1 se correlacionó positivamente con RI, mientras que en el fenotipo Th2, no hubo diferencias significativas en los niños con y sin obesidad (Pacifico *et al.,* 2006). Nuestros resultados confirman el predominio de células Th1 circulantes en obesidad. Además se encontró una disminución de las células Th2

CD3$^+$CD4$^+$IL4$^+$, C3$^+$CD4^{int}IL4$^+$, C3$^+$CD4$^-$IL4$^+$ y C3^{int}CD4$^-$IL4$^+$ en los individuos obesos. Estos resultados muestran que la desregulación metabólica causada por la obesidad puede ser afectada por las células T CD3$^+$CD4$^+$ y el desequilibrio entre células Th1 y Th2 puede ser un componente fisiopatológico de la obesidad.

Por otra parte en otros estudios, en varios grupos de pacientes los monocitos CD14$^+$ y los neutrófilos han sido identificados como mediadores inflamatorios entre la obesidad y sus complicaciones metabólicas y cardiovasculares (Viardot, *et al.,* 2012, Ghanim *et al.,* 2004; Schipper *et al.,* 2012). Por el contrario, en este estudio se observó un aumento en las subpoblaciones de monocitos-macrófagos CD68$^+$CD14$^-$, CD16$^+$CD14$^-$ y CD16^{low}CD14$^-$ en jóvenes con obesidad. Similar a nuestros resultados otros estudios muestran que la obesidad se caracteriza por un aumento en circulación de monocitos CD16$^+$ (Rogacev *et al.,* 2010), CD14^{dim}CD16$^+$ y CD14$^+$CD16$^+$ (Poitou *et al.,* 2011; Cottam *et al.,* 2002). Un aumento significativo en el subtipo de células CD16$^+$ también se ha descrito en sepsis, artritis reumatoide, infecciones, y se ha relacionado con eventos cardiovasculares en pacientes con enfermedad renal crónica (Ziegler-Heitbrock, 2007; Heine *et al.,* 2008; Rogacev *et al.,* 2011). Además las células CD16$^+$ muestran un fenotipo similar a macrófagos con una mayor capacidad presentadora de antígeno, alta afinidad endotelial y una potente capacidad para invadir las lesiones vasculares y secretar citocinas proinflamatorias (Rogacev *et al.,* 2010; Ziegler-Heitbrock, 2007). Con base en estas observaciones, se podría proponer a los monocitos CD16$^+$ como marcadores tempranos de la inflamación de bajo grado en la obesidad y sus comorbilidades relacionadas.

Un hallazgo importante de este estudio es que las mujeres con obesidad presentan un mayor número de células CD68$^+$CD14$^-$ en comparación con las de peso normal. Esto podría sugerir que el aumento de estas células es debido a la ganancia de peso, pero también puede ser influenciado por el género. Cabe mencionar que las mujeres con y sin obesidad no mostraron ninguna otra alteración metabólica como la DMT2. Se ha reportado que las mujeres tienen un menor riesgo cardiovascular antes de la menopausia en comparación con los hombres, pero este mecanismo de

protección en las mujeres no está completamente entendido, pero puede estar relacionado en parte, al efecto protector de los estrógenos endógenos sobre el riesgo cardiovascular en mujeres pre-menopáusicas. Por ello la prevalencia de los factores de riesgo en las mujeres debe ser mucho mayor que en los hombres para llevar a la aparición de la enfermedad cardiovascular a la misma edad que en los hombres, debido a que la enfermedad cardíaca coronaria aparece típicamente 10 años más tarde en mujeres (Knopp *et al.,* 2002; Gouva *et al.,* 2004). Se requieren más estudios para establecer la función de la subpoblación CD68^{+}CD14^{-} en el desarrollo de la obesidad y sus comorbilidades asociadas.

Varios estudios han reportado niveles incrementados de marcadores inflamatorios como CRP, IL-6, IL-8, MCP-1 y MIF en individuos con obesidad y DMT2, y se correlacionaron positivamente con el IMC y la circunferencia de la cintura (Khaodhiar *et al.,* 2004; Kim *et al.,* 2006; Dandona *et al.,* 2004). MIF se ha destacado como un importante regulador de la respuesta inmune innata y adaptativa inflamatoria (Calandra *et al.,* 2003), por lo que analizamos si podría ser inductor del perfil de citocinas Th1 debido al efecto que tiene sobre los macrófagos y células T. Sin embargo, no encontramos una relación de MIF con el perfil de citocinas proinflamatorias. Los niveles de expresión y circulantes de MIF pueden estar relacionados con variantes genéticas dentro de la región promotora de *MIF,* para ello buscamos esta relación en los grupos estudiados. Los resultados muestran un aumento en la expresión del mRNA de *MIF* en obesos en comparación con los de peso normal. Varios estudios han reportado la expresión del mRNA y los niveles séricos de MIF incrementados en personas con obesidad (Dandona *et al.,* 2004; Ghanim *et al.,* 2004; Church *et al.,* 2005; Kamchybekov *et al.,* 2012). En cuanto a los polimorfismos en obesidad han sido muy poco estudiados, en 2006, Sakaue *et al.,* encontraron que el STR -794 CATT$_{5-8}$ se asoció con la obesidad en una población japonesa. Es importante mencionar que este es el primer estudio que reporta la relación entre polimorfismos en el gen *MIF* y la expresión del mRNA de *MIF* en obesidad. Los resultados muestran un moderado incremento en la expresión del mRNA de *MIF* en los portadores de los genotipos -794 CATT$_{6,6}$ y -173C/C y el

haplotipos 6G de ambos polimorfismos estudiados en comparación a los otros genotipos y haplotipos en jóvenes con y sin obesidad. Además los portadores del genotipo 6,6 con obesidad tienen un incremento en la expresión del mRNA en comparación con los portadores del 6,6 con normopeso, aunque esta diferencia no es estadísticamente significativa. Previamente, se ha demostrado en ensayos *in vitro* que el repetido 6,6 regula la actividad transcripcional basal e inducida por estímulos, la cual aumenta casi proporcionalmente con el número de repetidos. En ensayos con un gen reportero se ha observado que el alelo $CATT_5$ se asocia con una baja actividad transcripcional a nivel basal y bajo estímulos que los alelos $CATT_6$ y $CATT_7$ (Baugh *et al.,* 2002; Renner *et al.,* 2012), pero se desconoce qué tipo de factor de transcripción regula la expresión de MIF a través de la región del promotor que contiene los repetidos CATT, mientras que portadores del alelo $CATT_7$ presentan altos niveles circulantes de MIF (Radstake *et al.,* 2005). Con respecto al polimorfismo -173 G>C regula la actividad transcripcional de *MIF* a nivel basal y bajo estimulo de una manera dependiente del tipo de célula (Donn *et al.,* 2002). Por otra parte, el alelo C se asocia con un aumento de los niveles circulantes de MIF (Donn *et al.,* 2002; Radstake *et al.,* 2005). Estos resultados apoyan lo observado en este estudio. Sin embargo, no se encontró una asociación entre la obesidad y los polimorfismos en el promotor del gen *MIF* con la expresión del mRNA en la población guerrerense estudiada. A diferencia de lo observado en otros estudios donde muestran una relación de MIF con la obesidad, la desregulación del metabolismo de la glucosa, RI y DMT2; sugiriendo un papel fisiopatológico importante de MIF y su contribución en el desarrollo de la inflamación crónica en la obesidad y su papel como probable blanco terapéutico para reducir el estado inflamatorio en la obesidad y sus comorbilidades.

Además, se conoce que en la obesidad se presenta la inflamación sistémica crónica de bajo grado, que se caracteriza por el incremento en los niveles sanguíneos de algunas citocinas y quimiocinas. Por lo que en este trabajo se analizó la relación entre las citocinas y la proteína C reactiva de alta sensibilidad (hsCRP) con la presencia de alteraciones metabólicas en los jóvenes son y sin obesidad. Tanto en

niños como en adultos, varios estudios muestran un incremento en los niveles séricos de IL-6, MCP-1 y CRP en individuos con obesidad (Khaodhiar *et al.,* 2004; Kim *et al.,* 2006; Schipper *et al.,* 2012; Breslin *et al.,* 2012). De igual forma, encontramos niveles séricos incrementados de IL-6 y hsCRP en jóvenes con obesidad general y abdominal en comparación con los de peso normal. Se conoce que durante la obesidad, la IL-6 se libera por el tejido adiposo visceral en la circulación portal y que la CRP se sintetiza principalmente en el hígado en respuesta a la estimulación por IL-6, lo que explicaría su aumento proporcional (Anty *et al.,* 2006). La CRP tiene un efecto en la amplificación de la respuesta inflamatoria y se utiliza como un marcador de la inflamación relacionada con la obesidad y es un predictor de eventos cardiovasculares y diabetes (Bisoendial *et al.,* 2009; Ridker 2007). En este estudio no se encontraron diferencias significativas en los niveles séricos de MIF, IL-1β, IFN-ɣ, IL-2, IL-12, IL-4 e IL-10 entre los grupos de estudio y con y sin anormalidades metabólicas. Sin embargo, estudios previos sobre los niveles séricos de estas citocinas son inconsistentes en individuos con obesidad, DMT2 y MetS. Se han reportado niveles séricos incrementados de IL-12, MIF, IL-1β, IL-4, IL-5, IL-10, IL-13, IFN-ɣ y TNF-α en individuos con obesidad (Suárez-Álvarez *et al.,* 2013; Leon-Cabrera *et al.,* 2013; Kamchybekov *et al.,* 2012; El-Wakkad *et al.,* 2013; Schmidt *et al.,* 2015), y elevados niveles circulantes de IL-12, IFN-ɣ, IL-4, IL-5 e IL-13 en personas con MetS (Surendar *et al.,* 2011). En otros estudios, se encontraron resultados inconsistentes para los niveles séricos de IL-10, mujeres obesas presentaron niveles incrementados de esta citocina (Eposito *et al.,* 2003) y niveles séricos disminuidos en individuos obesos, pero sin cambios en las concentraciones después de la pérdida de peso corporal en respuesta a la dieta (Manigrasso *et al.,* 2005). Por lo tanto, una producción desregulada de citocinas pro-inflamatorias y anti-inflamatorias parece jugar un papel importante en la fisiopatología de la obesidad y sus comorbilidades metabólicas asociadas.

En el presente estudio, los jóvenes con hipertrigliceridemia tuvieron niveles séricos más altos de hsCRP en comparación con los jóvenes sin esta dislipidemia. También, se encontró una correlación positiva entre las medidas de la adiposidad y los niveles sanguíneos de IL-6 y hsCRP, pero una correlación negativa con los niveles de IL-10.

Similarmente, otros estudios han reportado correlaciones positivas entre los niveles de IL-6 y CRP con el IMC, circunferencia de cintura y cadera, y el porcentaje de grasa corporal (Khaodhiar *et al.,* 2004; Utsal *et al.,* 2012; Paepegaey *et al.,* 2014). En conjunto estos estudios muestran consistentemente una relación entre los niveles circulantes de IL-6 y hsCRP con la adiposidad corporal, donde uno de los principales efectos de la IL-6 puede ser la inducción de la producción hepática de CRP, por lo tanto aumentan simultáneamente ambos marcadores que refleja una condición inflamatoria en los individuos con obesidad. Debido a que la hsCRP se encuentra incrementada en la obesidad abdominal y la hipertrigliceridemia, se puede considerar un mejor marcador de riesgo cardiometabólico en comparación con las citocinas. Por lo que se sugiere que la hsCRP, IL-6 e IL-10 son los principales marcadores inflamatorios relacionados con la obesidad y/o dislipidemia en jóvenes, que pueden ser útiles en la detección temprana del riesgo cardiovascular.

A continuación se propone un modelo de integración de los resultados de este trabajo de investigación en jóvenes con y sin obesidad. Se encontró un aumento en las concentraciones séricas de glucosa y del perfil lipídico (CT, TG, LDL-C) en los jóvenes con obesidad en comparación con los de peso normal, este mismo grupo presentó un incremento de las células T $CD3^{+}CD4^{+}$ circulantes que se polarizan hacia el fenotipo Th1 proinflamatorio, y una disminución de las Th2 antiinflamatorias. Además, los jóvenes obesos tuvieron un incremento en los niveles séricos de IL-6 y hsCRP y una disminución de los niveles séricos de IL-10. La expresión del mRNA de *MIF* se encontró incrementada en el grupo con obesidad y en los portadores de los genotipos -794 $CATT_{66}$ y -173C/C y del haplotipo 6G de los polimorfismos -794 $CATT_{5-8}$ y -173 G>C en el gen *MIF*, aunque esta diferencia no fue estadísticamente significativa, además no se observó una relación de estos polimorfismos con los niveles séricos de MIF en la población estudiada. En resumen podemos observar que en los jóvenes con obesidad hay una desregulación metabólica con respecto a los de peso normal y que en este proceso están implicados no solo células del sistema inmune innato y adaptativo sino también citocinas proinflamatorias y variantes en el gen de *MIF*, que en conjunto contribuyen a la fisiopatología de la

obesidad y finalmente llevan al desequilibrio metabólico y al desarrollo de enfermedades asociadas a la obesidad (Figura 3).

Figura 3. Modelo comparativo de componentes metabólicos e inflamatorios en jóvenes con normopeso y obesidad.

Los cambios inducidos por la obesidad en el número y la actividad de las células inmunes residentes en el tejido adiposo resultan en la activación de la respuesta inflamatoria local y sistémica, marcando la transición desde la simple adiposidad a comorbilidades asociadas a la obesidad. En nuestro estudio se encontró en los jóvenes con obesidad un incremento en el número de células de la respuesta inmune innata (monocitos-macrófagos $CD68^+CD14^-$, $CD16^+CD14^-$ y $CD16^{low}CD14^-$) y adaptativa (células $TCD3^+CD4^+$ y Th1) y una disminución de las células Th2. Sin embargo, no se encontraron diferencias significativas entre los niveles séricos de

citocinas que secretan estas células: MIF, IL-1β, IFN-ɣ, IL-2, IL-12, IL-4 e IL-10 en ambos grupos de estudio y de acuerdo con anormalidades metabólicas (Figura 4). La secreción de citocinas por estas células no necesariamente corresponde a los niveles séricos, y si estas células se encuentran en la etapa inicial de activación pueden progresar y amplificar posteriormente la respuesta inmune en los jóvenes con obesidad. Aunque desconocemos el tiempo de evolución de la obesidad, suponemos que es de reciente inicio, ya que la mayoría no presentan síndrome metabólico y más del 60% presentan obesidad clase I (30–34.9 kg/m^2), donde aún no hay un incremento importante de citocinas proinflamatorias. Además se ha reportado que la presencia de DMT2 favorece un perfil de citocinas Th1 en sujetos con DMT2-CAD con supresión del perfil de citocinas Th2 (Madhumitha, *et al.,* 2014), por esta razón es importante mencionar que los jóvenes obesos no presentan DMT2, solo 4 tienen glucosa alterada en ayuno, y con respecto al número de alteraciones metabólicas en los obesos, el 18% tiene 1 alteración, 41% con 2 alteraciones y el 38% con 3 o más alteraciones metabólicas. Puede ser que haya producción alterada de otros marcadores de inflamación, además de IL-6 y CRP en el grupo de obesos, pero a nivel local en el tejido adiposo, por lo que no se refleja a nivel sistémico. Además de la edad, número de alteraciones metabólicas y el tiempo de evolución de la obesidad, otros factores pueden contribuir a las diferencias en los resultados obtenidos en los estudios donde se miden los niveles de marcadores de inflamación entre personas obesas y delgadas, como el tamaño de muestra y los criterios de inclusión en cada estudio, así como la influencia racial entre poblaciones con diferente origen étnico. En conclusión los jóvenes con obesidad presentan mayor número de monocitos-macrófagos, células TCD3CD4 que se polarizan hacia el fenotipo Th1 y una disminución de las Th2, un aumento en los niveles séricos IL-6 y hsCRP, así como un leve incremento en la expresión del mRNA del *MIF* en comparación con los de peso normal, lo que sugieren que la desregulación metabólica causada por la obesidad puede ser modulada por las células del sistema inmune innato y adaptativo y citocinas proinflamatorias que en conjunto contribuyen a la fisiopatología de la obesidad.
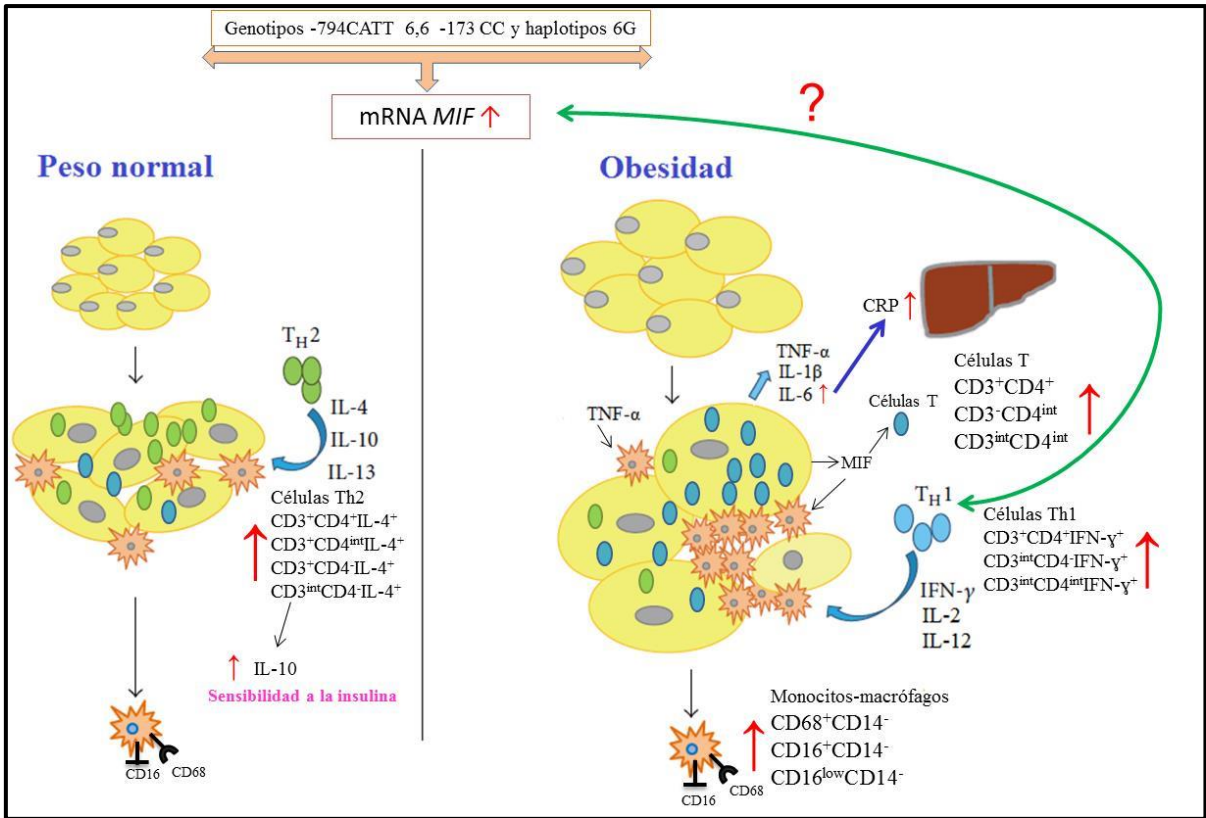
Figura 4. Modelo comparativo de componentes inflamatorios en jóvenes con normopeso y obesidad.

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Anexo

El factor inhibidor de la migración de macrófagos y su relación con la obesidad y la diabetes.

El factor inhibidor de la migración de macrófagos y su relación con la obesidad y la diabetes.

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Resumen. En diversos estudios se ha identificado que la obesidad y principalmente el aumento de adiposidad en la región abdominal, se asocia con inflamación de grado bajo, resistencia a la insulina (RI), homeostasis alterada de la glucosa y con sus comorbilidades tales como la diabetes mellitus tipo 2 (DMT2), la hipertensión, las dislipidemias y las enfermedades cardiovasculares. El factor inhibidor de la migración de macrófagos (MIF) es una citocina proinflamatoria involucrada en enfermedades autoinmunes e inflamatorias. Sin embargo, actualmente, se sugiere que el MIF está involucrado en el proceso inflamatorio que acompaña a la obesidad, así como en el control metabólico de las complicaciones asociadas a la obesidad. Los diferentes estudios muestran de manera consistente, el aumento en los niveles séricos del MIF en personas con obesidad, diabetes tipo 2 y en los diabéticos que presentan complicaciones microvasculares (la nefropatía, la retinopatía y el síndrome de pie diabético). La relación del MIF con la regulación del metabolismo de la glucosa y la apoptosis de las células β pancreáticas, así como la asociación de algunos polimorfismos funcionales en el promotor del gen del *MIF* con la obesidad y la diabetes. Esta revisión resume conocimientos basados en estudios clínicos y epidemiológicos sobre el papel del MIF en la obesidad y la diabetes tipo 2.

96

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Macrophage migration inhibitory factor and its relationship with obesity and diabetes.

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Abstract. Several studies have found that obesity and increased adiposity mainly in the abdominal region, are associated with low-grade inflammation, insulin resistance (IR), impaired glucose homeostasis and comorbidities such as type 2 diabetes mellitus (T2D) and cardiovascular disease. The macrophage migration inhibitory factor (MIF), is a proinflammatory cytokine involved in autoimmune and inflammatory diseases. However, currently it is suggested that MIF is involved in the inflammatory process associated with obesity and the metabolic control of the complications associated with obesity. Different studies show consistently, increased serum levels of MIF in subjects with obesity, type 2 diabetes and diabetics with microvascular complications (nephropathy, retinopathy and diabetic foot syndrome). The relationship of the MIF to the regulation of glucose metabolism and apoptosis of pancreatic β cells, and the association of some functional polymorphisms in the promoter of the *MIF* gene with obesity and diabetes.This review summarizes, the knowledge based on clinical and epidemiological studies on the role of MIF in obesity and type 2 diabetes.

INTRODUCCIÓN

La obesidad es una enfermedad crónica y multifactorial, cuya prevalencia va en aumento a nivel mundial. Este incremento se puede atribuir a la disponibilidad y al consumo de alimentos con alto contenido de grasa, en combinación con un estilo de vida sedentario (1, 2). En consecuencia, existe un sustancial aumento en las comorbilidades relacionadas con la obesidad, entre las que se incluyen, la resistencia a la insulina (RI), la homeostasis alterada de la glucosa, la diabetes mellitus tipo 2 (DMT2), la hipertensión arterial, las dislipidemias y las enfermedades cardiovasculares (ECV) (3-6).

Actualmente, se considera al tejido adiposo como un órgano endocrino activo, que secreta mediadores importantes de la inflamación, tales como la interleucina-6 (IL-6), el factor de necrosis tumoral-α (TNF-α), la resistina, la interleucina-8 (IL-8), la proteína quimioatrayente de monocitos-1 (MCP-1), la interleucina 1-β (IL-1β) y el factor inhibidor de la migración de macrófagos (MIF). La red de citocinas favorece la producción de reactantes de fase aguda como la proteína C reactiva (CRP), la haptoglobina y el fibrinógeno, que en conjunto contribuyen a un estado de inflamación crónica de grado bajo característico de la obesidad; además de que algunas de estas adipocinas se encuentran implicadas en el desarrollo de la RI y el síndrome metabólico (5, 7, 8).

El factor inhibidor de la migración de macrófagos (MIF) fue una de las primeras

citocinas proinflamatorias que se describió en 1966, como un factor soluble expresado por linfocitos T en respuesta a la hipersensibilidad tipo retardada y ejerce un efecto inhibitorio sobre la migración de los macrófagos *in vitro* (9, 10). Se han realizado estudios que demuestran que el MIF es un importante regulador de la respuesta inmune innata e inflamatoria (11). El MIF se produce por diferentes tipos de células y tejidos como, células T, macrófagos, monocitos, glándula pituitaria, fibroblastos, células endoteliales y adipocitos (12 -14). Estimula la expresión y secreción de citocinas proinflamatorias como son el TNF-α, el interferón gamma (IFN-γ), la IL-1β, la IL-6, la interleucina-2 (IL-2) y la IL-8 y puede a su vez contraregular el efecto antiinflamatorio de los glucocorticoides (15-17). Se ha determinado que los niveles del MIF se encuentran incrementados en la artritis reumatoide (AR), la sepsis severa, la obesidad y la DMT2, siendo que estas enfermedades cursan con inflamación persistente de diferentes grados (18-21), es importante realizar estudios para tratar de dilucidar el papel del MIF en su desarrollo.

FACTOR INHIBIDOR DE LA MIGRACIÓN DE MACRÓFAGOS Y OBESIDAD

La inflamación constituye una respuesta fisiológica del organismo ante las infecciones o heridas, que tiene como fin el restablecimiento de la homeostasis. En general, se considera que dicha respuesta es beneficiosa debido a que proporciona protección controlada contra las infecciones. La obesidad se acompaña de inflamación crónica de grado bajo, y hasta el momento no se le ha atribuido un efecto positivo (3, 22). La obesidad altera la función endocrina y metabólica del tejido adiposo y lo lleva a un incremento en la liberación de ácidos grasos, hormonas y moléculas proinflamatorias, así como a una infiltración de monoci-

tos que contribuyen a las complicaciones asociadas a la obesidad. En el 2003, dos estudios independientes demostraron que la expansión del tejido adiposo se acompaña por una infiltración progresiva de monocitos en el tejido adiposo (TA). Weisberg y col. y Xu y col. reportaron que el TA en ratones obesos presenta infiltración de monocitos, lo cual se puede atribuir a la muerte de las células grasas hipertróficas y/o a una hipersecreción por parte del TA de citocinas proinflamatorias, tales como MIF, IL-6, TNF-α, IL-1β y MCP-1 (23, 24).

Los macrófagos son una fuente primaria del MIF *in vitro* e *in vivo* (25). El MIF se secreta en respuesta a estímulos inflamatorios como lipopolisacárido (LPS), TNF-α e IFN-γ (11, 25, 26). MIF puede actuar de manera autocrina y paracrina e inhibe el efecto inmunosupresor de los glucocorticoides, al promover la secreción de una variedad de citocinas proinflamatorias, como TNF-α, IL-2, IL-6, IL-8, IFN-γ, IL-1β, además de que inhibe a la citocina inmunomoduladora IL-10, lo que conlleva a la sobrexpresión del MIF y al desarrollo de un microambiente inflamatorio (11, 25-28). Roger y col., demostraron que los macrófagos MIF -/- tuvieron una baja respuesta al LPS, debido a la reducción en la actividad del NF-κB y la producción del TNF-α (29). También se ha determinado que el MIF endógeno, regula la inmunidad innata a través de la sobre regulación de la expresión del receptor tipo toll 4 (TLR4), el receptor de IL-1 (IL-1R) y el receptor para TNF (TNFR) (29, 30). Además, el MIF disminuye la apoptosis dependiente de p53 lo que conlleva a un incremento de la vida media de los macrófagos activados, y por lo tanto a una amplificación de la respuesta inflamatoria (31). Debido a su funcionalidad y prominente papel en la biología de los macrófagos, así como su propiedad quimioatrayente (32), el MIF puede promover el reclutamiento de macrófagos y células T al tejido adiposo, por lo

que se ha implicado en la inflamación asociada a la obesidad y sus complicaciones metabólicas relacionadas.

Los adipocitos humanos y murinos, también secretan el MIF (13, 33). Los niveles de ARNm del MIF en células mononucleares de sangre periférica de pacientes obesos y los niveles séricos de MIF se encuentran incrementados en obesidad (19, 34). Alvehus y col. reportaron que la expresión del MIF fue mayor en tejido adiposo visceral humano, lo que sugiere que el MIF es sobre-regulado por la grasa intra-abdominal, metabólicamente perjudicial, así mismo observaron una asociación positiva entre el porcentaje de grasa corporal y la expresión de MIF en el tejido adiposo visceral, por lo que sugirieron que el MIF puede promover la acumulación de triglicéridos. Siendo posible que la alta expresión del MIF facilite el almacenamiento de grasa corporal en el depósito metabólicamente desfavorable (35). Saksida y col., mostraron aumento en la producción local y sistémica de MIF en ratones C57BL/6 alimentados con una dieta alta en grasa, que se correlacionó con la ganancia de peso y el desarrollo de la intolerancia a la glucosa (36). Varios estudios indican que el MIF se encuentra posicionado en la parte superior de la cascada de señalización inflamatoria y que la deficiencia del MIF beneficia casi todas las condiciones con procesos inflamatorios; pero también reduce la capacidad de las células de producir TNF-α, IL-1, IL-6, interleucina-17 (IL-17) e interleucina-23 (IL-23) (37, 38). Particularmente, la deficiencia del MIF reduce el tamaño del adipocito y la inflamación del tejido adiposo, lo cual indica un papel crucial del MIF en la inflamación y disfunción metabólica del tejido adiposo asociado a la obesidad (39).

En un estudio para evaluar el efecto del ejercicio sobre la obesidad y la enfermedad de hígado graso no alcohólico, realizado en ratones y en cultivos de adipocitos humanos, se encontró incremento en la expresión hepática de MIF, en los ratones que fueron sometidos a ejercicio. Los cultivos de hepatocitos humanos, que fueron sometidos a tratamiento con MIF, aumentaron la fosforilación de AMP activada por la proteína cinasa y la actividad de la acetil-CoA carboxilasa, además de que aumentó la oxidación de los lípidos. Estos resultados sugieren que el ejercicio incrementa la expresión de MIF en el hígado y puede prevenir la esteatosis activando la vía AMPK (40).

Niveles séricos del Factor Inhibidor de Macrófagos en obesidad

Diversos estudios epidemiológicos relacionan los niveles sanguíneos del MIF con la obesidad. Dandona y col., reportaron una correlación entre los niveles séricos de MIF y el índice de masa corporal (IMC), encontrando que los individuos con obesidad con un IMC de 37,5 $\pm$ 4,9 kg/m^2 tienen concentraciones plasmáticas del MIF significativamente incrementadas (2,8 $\pm$ 2,0 ng/mL), en comparación con los individuos de peso normal con un IMC de 22,6 $\pm$ 3,4 kg/m^2 (MIF: 1,2 $\pm$ 0,6 ng/mL) (19). Similarmente, los niveles séricos del MIF se encontraron incrementados en adolescentes con sobrepeso en comparación con los de peso normal (MIF; mediana: 964,6 pg/mL, rango intercuartil: 590,3-2019,46 vs. 562,7 pg/mL, rango intercuartil: 430,6-813,76), y estos se correlacionaron positivamente con marcadores de inflamación y obesidad (34). Se ha demostrado que en la obesidad, la expresión del ARNm del MIF está sobre-regulada en 60% de las células mononucleares de sangre periférica y que el aumento en los niveles séricos y el ARNm del MIF en estas células se asocian con el IMC, ácidos grasos libres y la tolerancia disminuida a la glucosa (13, 18, 19, 41).

También se ha determinado el efecto de algunos medicamentos y de la reducción de peso corporal sobre los niveles sanguí-

neos del MIF. El tratamiento con la metformina, un fármaco anti-diabetes, disminuye las concentraciones plasmáticas del MIF de $2,3 \pm 1,4$ a $1,6 \pm 1,2$ ng/mL, en individuos con obesidad después de una intervención de 6 semanas. Después de retirar el tratamiento, los niveles del MIF regresaron a su valor inicial, lo cual indica un efecto dependiente de la metformina (19). Además, mediante un programa de pérdida de peso en personas con obesidad mórbida (IMC 43,0 $\pm 8,6$ kg/m^2) lograron reducir 14,4 kg de su peso corporal, y esto se asoció con una disminución en los niveles séricos del MIF y una mejoría de la función de las células β pancreáticas (42). En otro programa de pérdida de peso, se logró una reducción de peso de 4,4 kg, con una disminución del 67% en los niveles circulantes del MIF (43).

En un estudio realizado en indios Pima con obesidad y tolerancia normal a la glucosa, se observó que el tamaño de las células del tejido adiposo subcutáneo abdominal se asoció con las concentraciones de ARNm del MIF, en adipocitos y preadipocitos; además de que el incremento en los niveles de ARNm del MIF de ambos tipos de células se asoció con aumento en las concentraciones de insulina y glucosa plasmática, y con disminución de la acción de la insulina hepática y periférica (44).

Actualmente, existen numerosos estudios sobre genes asociados con el desarrollo de enfermedades poligénicas, como la obesidad, la diabetes, la artritis reumatoide, y la enfermedad cardiovascular, entre otras. Sin embargo, no ha sido posible identificar consistentemente a los genes o variantes de los mismos que se asocian con el riesgo o susceptibilidad para estas patologías. Aunque existen pocos estudios de asociación con el gen del *MIF,* se ha determinado la relación del polimorfismo funcional rs5844572 (-794 [CATT]$_{5\text{-}8}$) en la región promotora de este gen con varias enfermedades, particularmente el tetranucleótido

repetido localizado en la posición -794 se ha estudiado en población japonesa, encontrando que los alelos 6-, 7- y 8-CATT se asocian con la obesidad (45), pero aún no se conoce su relación con los niveles de la proteína.

FACTOR INHIBIDOR DE LA MIGRACIÓN DE MACRÓFAGOS Y DIABETES

Estudios epidemiológicos sugieren una relación entre el MIF y la homeostasis de la glucosa. Algunos investigadores se han enfocado a estudiar si el MIF es un factor causal de la desregulación del metabolismo de la glucosa, al afectar la producción de la insulina en las células β pancreáticas y/o en las células blanco de la insulina.

Papel del Factor Inhibidor de Macrófagos en el metabolismo de la glucosa

En estudios realizados *in vitro,* Waeber y col., mostraron la primera evidencia del papel del MIF en el metabolismo de la glucosa, encontrando que el MIF se produce en las células β pancreáticas y se localiza con la insulina en los gránulos secretores. La producción del MIF fue dependiente de la glucosa, regulando la liberación de la insulina de manera autocrina. Además, se observó que mediante la inmunoneutralización del MIF se disminuye la secreción de insulina inducida por la glucosa, en contraste a la exposición de MIF recombinante exógeno, que potenció la liberación de la insulina de los islotes pancreáticos (46). En estos estudios se sugiere que una disminución progresiva de la secreción del MIF dentro de los islotes de Langerhans, puede contribuir a la disfunción de las células β, a la disminución en la secreción de insulina y al incremento a nivel sistémico del MIF asociado con DMT2, lo cual puede representar un mecanismo fisiopatológico compensatorio para mejorar este defecto (46). Church y col., reportaron resultados similares en per-

sonas con obesidad que se sometieron a un programa de pérdida de peso, en este estudio se encontró que el incremento del MIF se asoció con disminución de la función de las células β en personas con hiperglucemia en ayuno, lo que apoya la hipótesis de que la acción disminuida de MIF en los islotes de Langerhans, puede contribuir a la disfunción de las células β en individuos con prediabetes. Esto se fortalece con el hallazgo en personas que tenían disminución sustancial en los niveles del MIF, que también presentaron aumento de la insulina plasmática, en comparación con los individuos que tuvieron un cambio mínimo en los niveles del MIF (42).

También se ha demostrado que MIF recombinante aumenta la apoptosis de los islotes pancreáticos tras la exposición a ácido palmítico o glucosa. El MIF potencia la disminución de la función de las células de los islotes inducida por nutrientes, según lo revelado por una menor tasa de oxidación de la glucosa, el contenido de ATP, y la despolarización de la membrana mitocondrial, favoreciendo la apoptosis mitocondrial. Los investigadores proponen que el silenciamiento del MIF puede mantener la integridad del páncreas endocrino (47).

En infecciones microbianas agudas, se puede inducir la respuesta inflamatoria sistémica del huésped, que frecuentemente se asocia con el incremento del catabolismo de la glucosa. La hiperglucemia transitoria y la RI normalmente ocurren primero, pero esto puede ser seguido por un estado persistente de la producción de lactato y acidosis metabólica, la disminución del glucógeno y la hipoglucemia (48). Benigni y col., reportaron en miotubos del músculo esquelético de la rata, que la administración de un anticuerpo anti-MIF suprimió la hipoglucemia inducida por el TNF-α e incrementó la síntesis de la fructosa 2,6-bifosfato, lo cual incrementa la glucólisis en las células del músculo esquelético. Esto sugiere que

los niveles séricos de MIF pueden incrementar la disposición de glucosa en el músculo esquelético. Así, el anticuerpo anti-MIF previene estos efectos en ratones TNF-α^{-/-} a los que se les administró endotoxina bacteriana, lo cual confirma la contribución del MIF a los cambios metabólicos inducidos por la inflamación (48).

Por otra parte, se ha demostrado *in vitro*, que la glucosa y la insulina regulan la expresión de MIF en adipocitos (7). El tratamiento de adipocitos 3T3-L1 con el TNF-α induce la secreción del MIF, sugiriendo que el TNF-α puede regular la producción de MIF en un ciclo de retroalimentación positiva durante la obesidad (49, 50). *In vivo,* los ratones MIF^{-/-} fueron hipo-respondedores a la administración del TNF-α y tuvieron normoglucemia comparado con los ratones control, lo cual indica que el MIF se requiere para la acción del TNF-α. Además, en los adipocitos tratados con el MIF recombinante, se ha demostrado que MIF regula el transporte de la glucosa mediado por la insulina y la transducción de señales del receptor de insulina. Del mismo modo, en respuesta al estrés inflamatorio, ratones MIF^{-/-} muestran una notable mejora en la captación de glucosa por el tejido adiposo comparado con la de los ratones control (51). Consistentemente, algunos estudios sugieren que el MIF afecta la homeostasis de la glucosa directa o indirectamente a través de la regulación del TNF-α. En otro estudio, encontraron que los ratones MIF-/- producen menos IL-6, TNF-α e IL-1β en comparación con los MIF+/+, lo que sugiere que el MIF puede contribuir a la patogénesis de la DMT2 al inducir la producción de citocinas proinflamatorias y/o al modular la función de los adipocitos (15). Aunque en otro estudio no se observó ningún efecto, cuando los ratones deficientes del MIF (MIF^{-/-}) y sus controles (MIF WT), fueron alimentados con una dieta alta en grasa, no se encontró diferencia en la ganancia

de peso o de tejido adiposo en los dos grupos de ratones, pero ambos grupos presentaron tolerancia disminuida a la glucosa y aumento en los niveles sanguíneos de insulina en ayuno (52).

Niveles séricos del Factor Inhibidor de Macrófagos en diabetes

La DMT2 es una enfermedad heterogénea caracterizada por la hiperglucemia que resulta del desarrollo progresivo de la RI acompañada por defectos en la secreción de insulina (53). Además de la predisposición genética, el riesgo de desarrollar la DMT2 aumenta con la edad, la obesidad, la hipertensión, la dislipidemia y la falta de actividad física (54). En varios estudios epidemiológicos, se ha observado una relación del MIF con el desarrollo de RI y DMT2 (18, 55-57).

En personas con DMT2, se ha encontrado aumento en los niveles séricos del MIF (20.7±13.3 ng/mL), en comparación con los controles no diabéticos (5,2 ± 3,0 ng/mL) (55). Se conoce que los indios Pima americanos, presentan una alta incidencia de DMT2, por lo que Vozarova y col., hicieron un estudio para conocer la influencia racial sobre los niveles sanguíneos del MIF, encontrando un aumento en los niveles del MIF en los indios Pima no diabéticos, en comparación con los caucásicos no diabéticos, determinando que este incremento se asocia con la alteración en la acción de la insulina, lo que apoya la idea de la asociación entre el MIF y la susceptibilidad para la RI y la DMT2 (18). En un estudio realizado por Herder y col., reportaron incremento significativo de los niveles del MIF, de la proteína C reactiva (CRP) y de la IL-6 en individuos con tolerancia disminuida a la glucosa y DMT2, comparado con los controles con normoglucemia. La asociación entre los niveles del MIF y la tolerancia disminuida a la glucosa y la DMT2 fue independiente de los otros marcadores inflama-

torios. A diferencia de la CRP y de la IL-6, hubo un incremento en los niveles séricos del MIF en el grupo con DMT2 con respecto al grupo con tolerancia disminuida a la glucosa, lo que sugiere que el incremento en los niveles del MIF precede al inicio de la DMT2 (56).

Además de los estudios que se han realizado sobre los niveles del MIF en la etiología de la DMT2, también se han analizado algunos polimorfismos en el gen del *MIF* con la susceptibilidad para desarrollar esta enfermedad. En el estudio MONICA/KORA, se analizaron 4 polimorfismos en el gen del *MIF* (rs755622, rs2070766, rs2070767 y rs1007888) y su relación con los niveles séricos de la proteína y el riesgo de desarrollar la DMT2. En este estudio se observó que el alelo C del polimorfismo rs1007888 se asoció con el incremento en los niveles séricos del MIF, mientras que el genotipo CC del mismo polimorfismo se correlacionó con la DMT2 en mujeres (57). Sin embargo, la asociación entre los niveles incrementados del MIF y la incidencia de la DMT2 fue significativamente más alta en las mujeres con obesidad, en comparación con las de peso normal. La relación triangular entre los genotipos, niveles séricos del MIF y la incidencia de la DMT2 en mujeres, sugiere que el MIF puede tener un papel causal en la etiología de la DMT2 y que el aumento en sus niveles circulantes puede conferir mayor susceptibilidad para la enfermedad (57). Aunado a estas variaciones genéticas, la diferencia entre género puede reflejar la influencia de las hormonas sexuales sobre el MIF a nivel transcripcional. Se ha observado que los estrógenos pueden regular la producción del MIF en monocitos y macrófagos de ratones y humanos, mediante la regulación transcripcional del factor nuclear κB (58). Recientemente, el polimorfismo rs1007888 también se asoció con una mayor susceptibilidad para la diabetes mellitus gestacional y el síndrome metabólico post-

parto (59). Un aumento en los niveles del MIF o de su receptor de superficie celular CD74, se han encontrado en diabéticos que presentan complicaciones microvasculares, como la nefropatía (55, 60), la retinopatía (55, 61, 62) y el síndrome de pie diabético (63).

En un estudio previo, realizado en la población del Occidente de México, se analizó la asociación de los polimorfismos funcionales rs5844572 (-794CATT$_{5-8}$) y rs755622 (-173G/C) con la artritis reumatoide (AR) y con los niveles séricos del MIF. Se encontró aumento en los niveles del MIF en los pacientes con AR en comparación con los controles. También, se observó asociación de los alelos de alta expresión -794CATT$_7$ y -173C en el gen del *MIF* con el inicio temprano de la AR y con la actividad clínica de la enfermedad. Además, ambos alelos presentaron un fuerte desequilibrio de ligamento (LD= 0,87, p <0,001) en la población mexicana, lo cual indica que estos alelos son segregados en bloque de una generación a otra y podrían conferir un riesgo similar (21, 64). Lo que sugiere que la proteína y el gen del *MIF* pueden contribuir al desarrollo de enfermedades autoinmunes, como la AR. Por otra parte, Vera y Meyer-Siegler, hicieron un meta-análisis sobre los estudios relacionados con el cáncer y el polimorfismo rs755622 (-173G/C) en el gen del *MIF*, y reportaron que el alelo -173C se asoció con alto riesgo para desarrollar cáncer, particularmente con tumores sólidos (OR= 1,89, IC95% 1,5-3,11, p=0,012) (65). Ambos polimorfismos funcionales en el promotor del gen del *MIF,* también se han relacionado con obesidad y diabetes (45, 57, 59).

La evidencia de los estudios epidemiológicos, básicos y clínicos muestran una relación del MIF con la obesidad, la desregulación del metabolismo de la glucosa, la resistencia a la insulina y la diabetes tipo 2; lo cual sugiere un papel fisiopatológico impor-tante del MIF y su contribución en el desarrollo de la inflamación crónica en la obesidad y su papel como probable blanco terapéutico, para reducir el estado inflamatorio en la obesidad y sus comorbilidades. También, se considera importante continuar realizando estudios de asociación de los polimorfismos en el gen del *MIF*, con las enfermedades que tienen como mecanismo fisiopatológico la inflamación; ya que se ha mostrado en algunas poblaciones que los polimorfismos rs5844572 y rs755622 pueden conferir mayor susceptibilidad para la obesidad, la diabetes y la artritis reumatoide. Sin embargo, aún no es claro si el incremento en los niveles del MIF lleva a la desregulación del metabolismo de la glucosa, la resistencia a insulina y la diabetes tipo 2, o si es un efecto secundario de la misma enfermedad, por lo que son necesarios más estudios para demostrar la participación del MIF en diferentes contextos fisiopatológicos.

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