UNIVERSIDAD AUTÓNOMA DE GUERRERO
FACULTAD DE CIENCIAS QUÍMICO BIOLÓGICAS
UNIDAD DE INVESTIGACIÓN ESPECIALIZADA EN MICROBIOLOGÍA
DOCTORADO EN CIENCIAS BIOMÉDICAS

"Polimorfismos del gen *MIF* (-794CATT$_{5\text{-}8}$ y -173G>C) en pacientes con esclerosis sistémica: asociación con la expresión de MIF-CD74 y la respuesta Th1/Th2/Th17"

T E S I S

QUE PARA OBTENER EL GRADO DE

DOCTORA EN CIENCIAS BIOMÉDICAS

PRESENTA:
M. en C. Christian Johana Baños Hernández

DIRECTOR DE TESIS:
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Dra. en C. Isela Parra Rojas

Chilpancingo, Gro., marzo de 2019.

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ACTA DE APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 01 días del mes de marzo del dos mil diecinueve, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado del Doctorado en Ciencias Biomédicas, para examinar la tesis titulada **"Polimorfismos del gen *MIF* (-794CATT$_{5-8}$ y -173G>C) en pacientes con esclerosis sistémica: asociación con la expresión de MIF-CD74 y la respuesta Th1/Th2/Th17 "**, presentada por la alumna Christian Johana Baños Hernández, 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 de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

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DIRECCIÓN 2018 - 2021

Este trabajo se realizó en el Instituto de Investigación de Ciencias Biomédicas del Centro Universitario de Ciencias de la Salud de la Universidad de Guadalajara, y el Laboratorio de Investigación en Obesidad y Diabetes de la Facultad de Ciencias Químico Biológicas de la Universidad Autónoma de Guerrero, en colaboración con el Servicio de Medicina Interna del Hospital General “Dr. Raymundo Abarca Alarcón” de Chilpancingo.

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En el periodo que cursó el Doctorado en Ciencias Biomédicas, la M. en C. Christian Johana Baños Hernández (CVU: 560787) fue beneficiada con la beca del CONACYT No.434856.

AGRADECIMIENTOS

Al **Dr. José Francisco Muñoz Valle**, por confiarme el desarrollo de este proyecto, por todas sus enseñanzas, por su paciencia, por su tiempo y por facilitarme todas las herramientas necesarias para culminar satisfactoriamente este proyecto. Cada uno de los logros académicos que he tenido sin duda son gracias a usted.

A la **Dra. Isela Parra Rojas**, por todo su apoyo durante desarrollo de este proyecto, sus sugerencias que contribuyeron al enriquecimiento del mismo, por su asesoramiento en todas las actividades del doctorado y por permitirme formar parte del laboratorio a su cargo.

Al **Dr. José Eduardo Navarro Zarza**, por todas las facilidades que me brindo durante la captación de pacientes, por todas sus enseñanzas en el campo clínico, por su confianza, por todo su apoyo, por su disposición, sus consejos, por estar presente en todos mis seminarios, y sobre todo por brindarme su amistad.

A mi comité de seguimiento: **Dra. Natividad Castro Alarcón, Dra. Edith Oregón Romero** y **Dr. Marco Antonio Leyva Vázquez** por todo su apoyo, por su disposición, por su tiempo y por sus valiosas aportaciones que enriquecieron esta tesis. Así como, a la **Dra. Berenice Illades Aguiar**, por su apoyo, sus comentarios y sugerencias durante los cursos de seminario de investigación, sin duda su labor fue fundamental en el desarrollo de este proyecto.

A la **Dra. Eugenia Flores Alfaro**, la **Dra. Luz del Carmen Alarcón Romero** y al **Dr. Julio César Lara Riegos** por aceptar formar parte de mi jurado, agradezco su tiempo e interés en este trabajo de tesis.

A todos los que forman parte de los laboratorios: **Instituto de Investigación en Ciencias Biomédicas (IICB)** y **Laboratorio de Investigación en Obesidad y Diabetes (LIOD)** por toda su ayuda, consejos, compartirme sus conocimientos y por qué hicieron que mi

estancia en estos laboratorios fuera muy grata. Gracias a todos los compañeros y amigos con los que conviví en estos laboratorios.

Al **Dr. Jorge Hernández Bello,** por todo su valioso apoyo en el desarrollo de este proyecto, por sus sugerencias, correcciones, enseñanzas, por compartir sus conocimientos conmigo, así como por brindarme su amistad.

A la mejor maestra que he tenido, la **Dra. Mirna Vázquez Villamar**, por tu paciencia, por compartir tus conocimientos conmigo, por las porras, porque siempre tienes tiempo para ayudarme, aunque estés muy ocupada, pero sobre todo por tu amistad.

A mis compañeros del doctorado, en especial **Marta y Jorge** por todos los buenos momentos que hemos compartido y por su amistad.

A mi amiga y compañera del doctorado **Lluvia,** por el apoyo incondicional, por los consejos y por la amistad que me has brindado.

A todos los que de una u otra manera pusieron un granito de arena para el desarrollo de ese proyecto… ¡GRACIAS!

DEDICATORIAS

Esta tesis está dedicada con todo mi cariño a las personas más maravillosas del mundo…MI FAMILIA

A mi esposo **Jonathan Dorantes de Jesús**, por tu amor, por tu comprensión, tu paciencia, tu apoyo incondicional, por que más que nadie estuviste conmigo en este proyecto y me animaste para seguir adelante, te amo.

A mi papá **Feliciano Baños Barragán** que me ve desde el cielo y sé que desde ahí ha velado por mí y está conmigo siempre. A mi mamá **Roberta Hernández Herrera** por darme la vida, por apoyarme incondicionalmente, por aconsejarme, por corregirme, por ser mi cómplice y darme ánimos para nunca rendirme, no hay palabras suficientes para agradecer todo lo que has hecho por mí.

A mis abuelitos **José, Gregorio y Justina** por ser mis segundos padres, por sus consejos, por cada una de sus enseñanzas, por su apoyo, por estar siempre conmigo y por su cariño. A mi abuelita **Felicitas**, que yo sé que desde el cielo celebra mis logros.

A mis hermanos **Bety, Maripaz, Jesús y Gregorio** por darme la fuerza para seguir adelante por medio de un abrazo o una sonrisa, por su apoyo, por ser los mejores hermanos, por creer en mí, por soportar mi carácter y por su cariño.

A mis tíos en especial **Ernesto, Conchis, Tomas, Gelacio y Betty,** por sus porras, sus consejos y todos los granitos de arena que aportaron para que yo pudiera cumplir uno más de mis sueños.

A todos mis familiares y amigos por todo lo que hicieron por mí, a todos que Dios los bendiga por siempre.

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RESUMEN

Antecedentes: El factor inhibidor de la migración de macrófagos (MIF) es una citocina pleiótropica con funciones proinflamatorias, la cual se ha sugerido que está involucrada en la patogénesis de la esclerosis sistémica (ES). El papel de los polimorfismos de *MIF* (-794 $CATT_{5-8}$ y -173 G>C), su expresión y la participación de su receptor de membrana CD74, siguen siendo escasamente conocidos en esta enfermedad.

Objetivo: Analizar la relación de los polimorfismos de *MIF* con la expresión de mRNA, los niveles séricos de MIF, su expresión en piel, el perfil de citocinas Th1/Th2/Th17 y la expresión de su receptor CD74 en ES.

Materiales y métodos: Se incluyeron 50 pacientes con ES y 100 sujetos control (SC). Los polimorfismos fueron genotipificados por PCR y PCR-RFLP. Los niveles de MIF se midieron por ELISA y el perfil de citocinas mediante el sistema BioPlex MagPix. La expresión del mRNA de *MIF* y *CD74* se cuantificó mediante la PCR en tiempo real. MIF y su receptor CD74 fueron evaluados por inmunohistoquímica en biopsias de piel.

Resultados: Los alelos -794 $CATT_7$ y -173*C del gen *MIF* y el haplotipo 7C se asociaron con la susceptibilidad para desarrollar ES ($p<0.05$). El haplotipo 7C se asoció con un aumento de la expresión del mRNA de *MIF* ($p= 0.03$) en ES. Sin embargo, la expresión del mRNA de *MIF* fue significativamente mayor en SC que en pacientes con ES ($p<0.05$); mientras que *CD74* no mostró diferencias significativas. Los niveles séricos de MIF fueron similares entre los pacientes con ES y SC ($p= 0.51$). Se observó un aumento de los niveles séricos de IL-1β e IL-6 en pacientes con ES, así como una correlación positiva entre los niveles séricos de MIF y los perfiles de citocinas Th1 y Th17. En las biopsias de piel de ES, la expresión de MIF y CD74 se encontró incrementada en queratinocitos y disminuida en células endoteliales. Por otro lado, la expresión de CD74 fue alta en los fibroblastos de los pacientes con esclerosis sistémica difusa (ESD).

Conclusiones: Los polimorfismos -794 $CATT_{5-8}$ y -173 G>C del gen *MIF* se asocian con susceptibilidad para ES en la población del sur de México y el haplotipo (7C) se relaciona con el aumento de la expresión del mRNA de *MIF*. Los resultados sugieren que MIF y CD74 podrían tener un papel importante en la patogénesis de la ES.

SUMMARY

Background: The macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine with proinflammatory functions that seems to be involved in the pathogenesis of systemic sclerosis (SSc). The role of *MIF* polymorphisms (-794 $CATT_{5-8}$ and -173 G>C), its expression and the participation of CD74, its membrane receptor, remain poorly understood in this disease.

Objective: To analyze the relationship of the *MIF* polymorphisms with mRNA expression, MIF serum levels, skin expression, the Th1/Th2/Th17 cytokine profile, and its receptor CD74 expression in SSc.

Materials and method: 50 SSc patients and 100 control subjects (CS) were enrolled in the study. The polymorphisms were genotyped by PCR and PCR-RFLP. MIF levels were measured by ELISA kit and cytokine profile using the BioPlex MagPix system. The *MIF* and *CD74* mRNA expression were quantified by and real-time PCR. MIF and its receptor CD74 were evaluated by immunohistochemistry in skin biopsies.

Results: The -794 $CATT_7$ and -173*C *MIF* alleles and the 7C haplotype were associated with SSc susceptibility ($p<0.05$). The 7C haplotype was associated with an increased *MIF* mRNA expression ($p=0.03$) in SSc. However, *MIF* mRNA expression was significantly higher in CS than in SSc patients ($p<0.05$); while *CD74* showed no significant differences. The MIF serum levels were similar between SSc patients and CS ($p=0.51$). In addition, an increase of IL-1β and IL-6 serum levels in SSc patients was found, as well as, a positive correlation between MIF serum levels and Th1 and Th17 cytokine profiles. In skin biopsies of SSc patients, the expression of MIF and CD74 increased in keratinocytes and decreased in endothelial cells. On the other hand, the staining of CD74 was high in fibroblasts of diffuse cutaneous systemic sclerosis (dcSSc) patients.

Conclusions: The polymorphisms -794 $CATT_{5-8}$ and -173 G> C of the *MIF* gene were associated with susceptibility to SSc from the southern Mexico population and the haplotype (7C) was related to the increase of *MIF* mRNA expression. The results suggest that MIF and CD74 could play an important role in the pathogenesis of SSc.

INTRODUCCIÓN

La esclerosis sistémica (ES) es una de las enfermedades autoinmunes sistémicas más complejas (Pattanaik, 2015). Las manifestaciones clínicas de la enfermedad son el resultado de tres procesos patológicos: lesiones microvasculares, alteraciones en el sistema inmune (autoinmunidad) y fibrosis (depósitos de colágeno) en piel y órganos internos (Barsotti, 2016). A pesar de que la fibrosis cutánea es la característica distintiva de la ES, también se presentan complicaciones de órganos viscerales como los pulmones, el tracto gastrointestinal, los riñones y el corazón determinan la progresión y gravedad de la enfermedad (Allanore, 2015). La afectación de órganos vitales conduce a la disminución de la supervivencia en los pacientes con ES (Pattanaik, 2015).

La ES también llamada esclerodermia es una enfermedad con manifestaciones clínicas y un pronóstico variable. Sin embargo, con base a la extensión de la afectación cutánea, los pacientes se agrupan principalmente en ES variedad cutánea limitada (ESL) y ES variedad cutánea difusa (ESD) (van den Hoogen, 2013; Pattanaik, 2015). Estos dos tipos de ES se diferencian principalmente en la extensión de piel afectada, la asociación con autoanticuerpos, y el patrón de afectación de órganos internos (Pattanaik, 2015).

Dada la heterogeneidad de los signos y síntomas clínicos de la ES, el Colegio Americano de Reumatología (ACR, por sus siglas en inglés *American College of Rheumatology*) y la Liga Europea Contra el Reumatismo (EULAR, por sus siglas en inglés *European League Against Rheumatism*), emitieron nuevos criterios de clasificación en el 2013. Estos criterios mejoraron la sensibilidad (96%) para el diagnóstico oportuno de la ES, además de que se incluyen los autoanticuerpos que se utilizan comúnmente para fines de diagnóstico (van den Hoogen, 2013).

La ES se considera una enfermedad poco frecuente, cuya prevalencia se estima entre 50 y 300 casos por cada millón de habitantes y una incidencia anual entre 2.3 y 22.8 casos por cada millón de habitantes al año. Sin embargo, la prevalencia e incidencia de ES varía dependiendo de la ubicación geográfica y origen étnico (Barnes, 2012). En México, no se han publicado cifras oficiales de la incidencia y prevalencia de la ES. No obstante, en un estudio realizado en el 2011 por Peláez-Ballestas y cols., en el cual se incluyeron 5 regiones diferentes de México (Ciudad de México, Nuevo León, Yucatán,

Sinaloa y Chihuahua) se reportó una prevalencia de 0.02% para la ES (Peláez-Ballestas, 2011).

El desarrollo de la ES depende del género, como sucede en la mayoría de las enfermedades reumáticas, es más frecuente en las mujeres que en los hombres, con una proporción que varía de 4:1 a 14:1 (Allanore, 2015). La incidencia más alta se ha observado generalmente en mujeres de mediana edad, entre los 40 y 60 años (Gu, 2008). Sin embargo, se ha reportado que también puede afectar a niños y ancianos, aunque con menor frecuencia (Katsumoto, 2011). A pesar de que esta enfermedad es menos común que otras enfermedades reumáticas, tiene una de las mayores tasas de mortalidad (Katsumoto, 2011).

La etiología de la ES aún es desconocida, sin embargo, se ven involucrados factores ambientales y genéticos en su desarrollo. El factor genético más importante de la ES son los genes del HLA de clase ll, siendo las variantes *HLA-DRB1*01, HLA-DRB1*11, HLA-A*30,* y *HLA-A*32* las que se han reportado con un alto grado de susceptibilidad genética para esta patología, mientras que los alelos *HLA-DRB1*07, HLA-B*57 y HLA-Cw*14* se han reportado como marcadores de protección para el desarrollo de la misma (Stern, 2015). En la población del sur de México, recientemente se evaluó la asociación de los receptores tipo inmunoglobulina (KIR) de las células NK que reconocen las moléculas HLA de clase I, los genotipos compuestos *KIR2DL2+/HLA-C1+* ó *KIR2DL2+/HLA-C2+* tuvieron una mayor frecuencia en pacientes con ES y se asoció con riesgo de para desarrollar ES (Machado-Sulbarán, 2019).

Se han identificado otros genes de riesgo para ES, tales como: *STAT4*, *PTPN22*, *IRF5, CD226, PDL4, CXCL8, HGF, CD247, IL-12R, IRAK1, IL-23R, TLR2, CTGF, KIR, MIF*, entre otros (Wu, 2006; Bossini-Castillo, 2015; Pattanaik, 2015). Las proteínas codificadas por estos genes están implicadas en vías moleculares en múltiples células del sistema inmune y células no inmunes (células endoteliales, fibroblastos y miofibroblastos) que están interconectadas y contribuyen a la progresión y exacerbación de la enfermedad (Zuber&Spertini, 2006). Las alteraciones funcionales en estas células dan lugar a tres cambios patológicos característicos de la ES: fibrosis cutánea progresiva y visceral, vasculopatía funcional y estructural, y alteraciones inmunológicas, que incluyen la

producción de autoanticuerpos e infiltrado de células mononucleares en los tejidos afectados (principalmente linfocitos T y macrófagos) (Zuber & Spertini, 2006; Katsumoto, 2011).

Las células T son un componente principal del infiltrado de la piel en pacientes con ES, en el estadio inflamatorio de la enfermedad (Gu, 2008). Varios autores han considerado que las citocinas del perfil Th2 estimulan la síntesis de colágeno e inhiben la producción de metaloproteinasas de matriz (MMPs, del inglés *matrix metalloproteases*) mediante la liberación de IL-4 e IL-13; pero las citocinas del perfil Th1 inhiben los depósitos de colágeno y mejoran la producción de MMP directamente mediante la liberación de IFN-γ y/o por contacto directo con los fibroblastos. Por lo tanto, las citocinas Th1 y Th2 juegan un papel importante en el desarrollo de la ES (Zuber&Spertini, 2006; Chizzolini, 2015).

Las citocinas Th1 controlan la velocidad de degradación del colágeno, regulando la síntesis de las MMPs y del inhibidor tisular de las metaloproteinasas (TIMP) (Raja&Denton, 2015). El TNF-α y el TNF-β, son citocinas proinflamatorias. Los macrófagos activados son la fuente más importante de TNF-α, pero las células T pueden hacer una contribución importante a su producción. TNF-α se considera a menudo como una citocina antifibrótica, ya que se ha descrito que puede inhibir la producción de colágeno tipo 1 en la piel, pulmón, sinovia, y fibroblastos conjuntivales, también es un potente inductor de la MMP-1 y MMP-3, y puede regular la disminución en la síntesis de TIMP-1 y TIMP-3 (Gu, 2008).

El perfil de citocinas Th2, que involucra las citocinas IL-4, IL-5 e IL-13, se ha relacionado con fibrosis en modelos animales y en enfermedades humanas. La IL-4 es una citocina que puede estimular directamente la síntesis de colágeno en los fibroblastos y parece potenciar la formación de TGF-β (Meloni, 2009). La IL-13 es una de las citocinas efectoras más importantes de la fibrosis en ES. La secreción de IL-13 se ha demostrado por inmunohistoquímica en muestras de piel de pacientes con ES en etapas tempranas y tardías (Yoshizaki, 2010).

Las células Th17 son una clase distinta de células T efectoras, su diferenciación es inducida por una elevada expresión de TGF-β, IL-6, IL-1β, IL-21 e IL-23. El perfil de

citocinas Th17 es IL-17A, IL-17F, TNF-α, IL-1, IL-6, IL-21, IL-22, IL-26 y el factor estimulador de colonias de granulocitos-monocitos (GM-CSF) así como la quimiocina CCL20. Este perfil de citocinas es principalmente proinflamatorio, y los efectos generales de estas citocinas podrían conducir a la inflamación y fibrosis exacerbada que es la característica distintiva de la ES. Aunque las células Th17 también están implicadas en la formación de centros germinales y en la producción de autoanticuerpos (Chizzolini, 2015; Rodríguez-Reyna, 2012).

Por otro lado, las células B son precursoras de las células plasmáticas productoras de autoanticuerpos y desempeñan un papel importante como células presentadoras de antígenos y productoras de citocinas. Existen indicios de un aumento de la activación de las células B en la ES, incluida la hipergammaglobulinemia, el aumento en el número de células B nativas, pero la disminución de las células B de memoria, la producción de autoanticuerpos y la sobreexpresión de CD19. Estos hallazgos sugieren que las células B pueden tener un papel importante en la patogénesis de la ES (Gu, 2008). En modelos murinos de fibrosis, la deficiencia de CD19, una molécula de transducción de señales de la célula B, resulta en una disminución de la fibrosis, lo cual, sugiere que las células B también pueden contribuir a la fibrosis. Las células B activadas además producen IL-6 y IL-10, ambas citocinas pueden promover una respuesta inmune Th2 predominante que induce la síntesis de colágeno. La producción de IL-6, así como la producción de TGF-β por células B activadas, también pueden inducir directamente la fibrosis de tejidos en pacientes con ES (Zuber&Spertini, 2006).

MIF es un regulador crítico de la respuesta inmunitaria, induce la expresión de citocinas y otros mediadores inflamatorios, incluyendo TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-12, óxido nítrico, MMPs específicas y productos de la cascada del ácido araquidónico. MIF también regula la expresión de receptores de la respuesta inmune innata como el receptor tipo Toll 4 (TLR4) y sensores microbianos especializados tales como dectina-1. Además de su función inmunológica, MIF promueve la supervivencia celular, la señalización de antioxidantes, la angiogénesis y la reparación de heridas, mientras que disminuye la senescencia celular, suprimiendo la activación inducida de la apoptosis dependiente de p53 (Calandra&Roger, 2003; Nguyen, 2003). Así también, se ha establecido que MIF está

implicado en la patogénesis de diferentes enfermedades autoinmunes incluyendo: esclerosis múltiple (EM), artritis reumatoide (AR), lupus eritematoso generalizado (LEG), artritis psoriásica (AP) y ES (Selvi, 2003; Llamas-Covarrubias, 2013; Morales-Zambrano, 2014; De la Cruz-Mosso, 2014; Baños-Hernández, 2019).

El gen de *MIF* en el genoma humano está localizado en el cromosoma 22 (22q11.2), compuesto por tres exones cortos de 172, 107 y 66 pares de bases, además de dos intrones de 188 y 94 pares de bases. Su región reguladora 5' contiene numerosas secuencias consenso para la unión de factores de la transcripción, como la proteína activadora 1 (AP-1), GATA-1, el factor de transcripción nuclear kappa B (NF-κB) y CREB (Calandra&Roger 2003).

Dos polimorfismos han sido identificados en el promotor del gen *MIF*, el primero es la repetición corta en tándem (STR) -794 $CATT_{5-8}$ (*rs5844572*) la cual es una repetición en microsatélite en la posición -794 pb rio arriba del gen *MIF;* la longitud de las repeticiones se correlacionan con la expresión del gen, lo cual se explica debido a que el factor de transcripción ICBP90 (también conocido como UHRF1) y Pit1 se unen a estas repeticiones, regulando positivamente la expresión de este gen (Yao, 2016). Un segundo polimorfismo reportado es el polimorfismo de nucleótido único (SNP) -173 G>C *(rs755622)* en la posición -173 rio arriba del gen *MIF* en el cual hay un cambio de guanina (G) por citocina (C), el alelo -173*C se asocia con niveles altos de MIF en la circulación, debido a que forma un sitio de unión para el factor de transcripción de la proteína activadora 4 (AP-4). Estos dos polimorfismos se han asociado en diferentes poblaciones con el incremento de la susceptibilidad al desarrollo de autoinmunidad y la severidad de las manifestaciones clínicas de enfermedades autoinmunes como AR, AP, LEG y EM (Calandra&Roger 2003).

El principal receptor de MIF es CD74 (cadena invariante, Ii), que es una glicoproteína transmembranal no polimórfica, que se expresa en células presentadoras de antígeno, incluyendo macrófagos y células B. Inicialmente se identificó como una molécula asociada con el procesamiento de las proteínas del MHC clase II (Schröder, 2016). Sin embargo, se demostró que CD74 actúa como receptor de membrana de alta afinidad ($\sim 9\times10^{-9} K_d$) para MIF (Leng et al., 2003). Una pequeña proporción de CD74 se modifica

mediante la adición de sulfato de condroitina (CD74-CS), y esta forma de CD74 se expresa en la superficie celular (Gore, 2008). Aunque la unión de MIF a CD74 es esencial para desencadenar la transducción de señales intracelular, se descubrió que no es suficiente, ya que el dominio intracelular de CD74 es de sólo 46 aminoácidos de longitud y carece de homología con tirosina cinasas, serina/treonina cinasas, dominios de interacción diferentes de cinasas o proteínas de unión a nucleótidos. Por lo cual, CD74 depende de la interacción con co-receptores, que mediante sus dominios citoplásmicos sean capaces de reclutar los componentes necesarios para activar la señalización (Shi, 2006; Schwartz, 2009).

Se ha descrito a CD44 como el principal co-receptor de CD74-CS. Los estudios muestran que en las células B, MIF inicia una cascada de señalización después de la unión al complejo CD74-CD44. Esta cascada de señalización implica a las cinasas Syk y Akt. Las cinasas Syk pertenece a la familia de las proteínas tirosina cinasas ZAP-70 y desempeña un papel crucial en el desarrollo de las células B. Además, se ha demostrado que Syk se requiere para la activación de Akt de una manera dependiente de fosfatidilinositol 3-cinasa (PI3K). La activación de Akt promueve una serie de respuestas celulares que están asociadas con la división celular, incluyendo el aumento en el tamaño de células, la supresión de la apoptosis, la inactivación de inhibidores del ciclo celular, y la expresión génica de citocinas. A nivel molecular, la expresión de Akt se correlaciona con el aumento en la función NF-kB, incluyendo la sobre regulación de *Bcl-xL* y *Bcl-2*, que conduce a una supresión de la apoptosis tanto *in vitro* como *in vivo* (Gore, 2008).

Por otro lado, en fibroblastos quiescentes el complejo MIF-CD74-CD44 induce una rápida (30 minutos) y sostenida (24 horas) fosforilación de la vía de señalización ERK1-ERK2-MAPK, lo que favorece la proliferación celular. Este mecanismo es dependiente de la cinasa A y está asociado a un incremento en la actividad enzimática de la fosfolipasa A2 (PLA2). La PLA2 es un importante enlace en la activación de la cascada proinflamatoria, resultando en la producción de ácido araquidónico y posteriormente en prostaglandinas (Bloom&Al-Abed 2014).

Otros co-receptores importantes son CXCR2 y CXCR4 que median los mecanismos de señalización que conducen a una variedad de respuestas biológicas. Esta variedad de respuestas biológicas incluye la regulación de la quimiotaxis, la proliferación, la angiogénesis y la aterogénesis, con efectos en la sepsis, la inflamación general, las enfermedades autoinmunes, las enfermedades cardiovasculares, y el crecimiento, la supervivencia y la metástasis en cáncer (Schwartz, 2009). Un estudio reciente, sugiere que MIF es un ligando alternativo de CXCR7 y que induce la internalización de CXCR7, de manera independiente de CXCR4, y la activación de la señalización ERK1/2 y ZAP-70, además de participar en la migración de linfocitos B, de manera dependiente de CXCR4 (Alampour-Rajabi, 2015).

Por otro lado, la asociación entre MIF y la ES fue descrita por primera vez por Selvi y cols., en el 2003. En su investigación, los autores observaron que las concentraciones de MIF en suero y en cultivos de fibroblastos de pacientes con ESD son significativamente más elevadas que en los controles. Además, detectaron la expresión de MIF en biopsias de piel de pacientes con ES, principalmente en queratinocitos, infiltrado de células mononucleares y fibroblastos, esto mediante tinción por inmunohistoquímica (Selvi, 2003).

Posteriormente, Wu y cols., reportaron por primera vez la asociación del polimorfismo -173 G>C de *MIF* con ES en población caucásica de Estados Unidos y Canadá; siendo el alelo -173*C de *MIF* más frecuente en los pacientes con ESD. Asimismo, observaron que los fibroblastos portadores del haplotipo 7C de *MIF* producían mayores niveles de MIF que los portadores de haplotipos distintos al 7C (Wu et al., 2006). Por su parte, Bossini-Castillo y cols., en el 2011 confirman la asociación del polimorfismo -173 G>C del gen *MIF* con ES en población europea, principalmente con el subgrupo de pacientes con ESD (Bossini-Castillo, 2011). En esta misma población, posteriormente, se describió una asociación significativa del polimorfismo -173G>C del gen *MIF* con la susceptibilidad para desarrollar hipertensión arterial pulmonar (HAP) en pacientes con ESD (Bossini-Castillo, 2017). Con base a los estudios anteriores, se sugiere al SNP -173 G>C de *MIF* como un marcador genético prometedor para esta enfermedad.

Becker y cols., observaron mayores niveles séricos de MIF en pacientes con ESD en comparación con pacientes con ESL y SC. Además, revelaron que los pacientes con HAP y úlceras digitales recurrentes mostraban niveles más elevados de MIF que los pacientes sin estas manifestaciones (Becker, 2008). Stefanantoni y cols., también reportaron el incremento en los niveles séricos de MIF en pacientes con HAP secundaria a ES en comparación con pacientes con ES sin HAP (Stefanantoni, 2015).

Kim y cols., confirmaron que los niveles séricos de MIF son significativamente mayores en los pacientes con ES que en los controles sanos. Además, observaron que MIF liberado por las células T activadas y fibroblastos dérmicos, disminuye la apoptosis de los fibroblastos dérmicos a través de la activación de las vías de señalización ERK, Akt y Bcl-2. Por lo que concluyeron que MIF podría estar asociado con la fibrosis excesiva en ES (Kim et al., 2008).

En el 2015, Corallo y cols., sugieren que MIF podría tener un papel importante en la patogénesis de la ES, ya que observaron mayores niveles séricos y de expresión de mRNA de esta citocina en los pacientes en comparación con los controles. Asimismo, en las biopsias de piel, se confirmó la expresión elevada de MIF en queratinocitos, fibroblastos, endotelio y glándulas sebáceas/sudoríparas de la piel afectada de pacientes con ESL/ESD. Sin embargo, no se encontraron diferencias al analizar los receptores de MIF, CD74/CD44 (Corallo, 2015).

Con base a lo anterior, fue de nuestro interés conocer la asociación del STR **-**794 $CATT_{5–8}$ y del SNP -173 G>C en el gen *MIF* con su expresión (proteína y mRNA) y su receptor CD74, así como con la respuesta Th1/Th2/Th17 en pacientes con ES del sur de México.

CAPÍTULO I

"*Macrophage migration inhibitory factor polymorphisms are a potential susceptibility marker in systemic sclerosis from southern Mexican population: association with MIF mRNA expression and cytokine profile*"

Clinical Rheumatology
https://doi.org/10.1007/s10067-019-04459-8

ORIGINAL ARTICLE

Macrophage migration inhibitory factor polymorphisms are a potential susceptibility marker in systemic sclerosis from southern Mexican population: association with *MIF* mRNA expression and cytokine profile

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Received: 7 November 2018 / Revised: 12 January 2019 / Accepted: 28 January 2019

Abstract

Introduction Systemic sclerosis (SSc) is a complex autoimmune disease, characterized by microvascular lesions, autoimmunity, and fibrosis. It is suggested that MIF participates in the amplification of the proinflammatory process in SSc; moreover, the promoter polymorphisms − 794 $CATT_{5–8}$ (rs5844572) and − 173G>C (rs755622) in the *MIF* gene have been associated with an increase in MIF serum levels in several autoimmune diseases. The aim of this study was to analyze the relationship of the − 794 $CATT_{5–8}$ and − 173G>C *MIF* polymorphisms with mRNA expression, MIF serum levels, and the Th1/Th2/Th17 cytokine profile in SSc.

Materials and methods A case-control study was carried out that included 50 patients with SSc and 100 control subjects (CS). Both polymorphisms were genotyped by PCR and PCR-RFLP. MIF levels were measured by ELISA kit. The cytokine profile and the *MIF* mRNA expression were quantified by BioPlex MagPix system and real-time PCR, respectively.

Results An association between the − 794 $CATT_7$ and − 173*C *MIF* alleles and the 7C haplotype with SSc susceptibility was found ($p < 0.05$). Also, the 7C haplotype was associated with increased *MIF* mRNA expression ($p = 0.03$) in SSc. In addition, an increase of IL-1β and IL-6 serum levels in SSc patients was found as well as a positive correlation between MIF serum levels and Th1 and Th17 cytokine profiles.

Conclusion The *MIF* 7C haplotype is a susceptibility marker for SSc in the southern Mexican population and is associated with *MIF* mRNA expression. Moreover, there is a positive correlation between MIF serum levels and Th1 and Th17 inflammatory response in SSc.

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Keywords Cytokine profiles Th1/Th2/Th17 · MIF · MIF mRNA expression · Promoter polymorphisms · Systemic sclerosis

Introduction

Systemic sclerosis (SSc), also referred to as scleroderma, is an autoimmune disease characterized by three pathological processes: microvascular lesions, impaired immune response, and fibrosis in skin and internal organs [1]. The heterogeneity of the clinical characteristics in SSc allows classifying patients based on their skin condition in limited cutaneous (lcSSc) and diffuse cutaneous SSc (dcSSc), the latter with worse prognosis [2]. Although SSc is a rare disease, it has one of the highest mortality rates among rheumatic diseases [3].

The etiology of SSc remains unknown, but it has been associated with several risk factors such as genetic and

Published online: 12 February 2019

environmental factors. The most important genetic factors are the HLA class II genes (*HLA-DRB1*01*, *HLA-DRB1*11*, *HLA-A*30*, and *HLA-A*32*), which have been associated with disease susceptibility in different populations [4]. Additional other genes outside the *HLA* region have been identified that contribute to the susceptibility and prognosis of SSc, including the *MIF* gene, which codes for a protein of the same name [5, 6].

MIF is an immunoregulatory cytokine that contributes to the pathogenesis of autoimmunity, infectious diseases, and cancer [7]. In addition, MIF promotes the survival of different types of cells such as dermal fibroblasts, by inhibiting apoptosis induced by p53 activation and inducing the secretion of proinflammatory cytokines such as TNFα, IL-1β, IL-2, IL-6, IL-8, and IL-12 [8, 9]. The *MIF* gene is located at the 22q11.23 locus and is made up of three exons and two introns [7]. Several polymorphisms have been described within the *MIF* promoter region, including the STR (short tandem repeat) −794 $CATT_{5–8}$ and the SNP (single nucleotide polymorphism) −173G>C, which have been associated with different autoimmune diseases such as rheumatoid arthritis (RA), psoriatic arthritis (PsA), systemic lupus erythematosus (SLE), and multiple sclerosis (MS) in the Mexican population [10–13].

The presence of a higher number of repetitions of STR −794 $CATT_{5–8}$ correlates with higher expression of *MIF* mRNA [14], which is explained by the fact that the transcription factor ICBP90 binds to these repetitions, regulating positively the expression of this gene [15]. With respect to SNP −173G>C, the C allele also is associated with higher MIF levels in serum and synovial fluid in RA patients [7, 16], most likely due to linkage disequilibrium with the high expression −794 $CATT_7$ allele.

In 2003, Selvi et al. reported for the first time that serum concentrations of MIF in patients with dcSSc were significantly higher than those in controls. Then, MIF expression was detected in skin biopsies of patients with SSc by immunohistochemical staining [5]. Two independent studies have reported that a functional *MIF* promoter polymorphism (−173G<C) was strongly associated with dcSSc [17, 18]. In addition, Becker et al. revealed that MIF can contribute to vasculopathy in the SSc [19]. On the other hand, it was reported that MIF stimulates the process of excessive fibrosis in SSc, increasing the proliferation of fibroblasts and collagen synthesis [20], and decreasing the apoptosis of dermal fibroblasts [9].

Despite the above-mentioned findings, the role of MIF in the pathogenesis of SSc remains poorly understood. The aim of this study was to evaluate the association of the *MIF* polymorphisms (−794 $CATT_{5–8}$ and −173G>C) with the expression of MIF and its correlation with Th1, Th2, and Th17 cytokine profile in patients with SSc from southern Mexican population.

Material and methods

Subjects

A case-control study was conducted with two study groups: The first group consists of 50 patients with SSc classified according to the 2013 American College of Rheumatology/European League Against Rheumatism classification criteria for SSc [2]. They were enrolled from Rheumatology Department at Hospital General de Chilpancingo "Dr. Raymundo Abarca Alarcón," Chilpancingo de los Bravo, State of Guerrero, Mexico. The modified Rodnan index Total Skin Score (TSS) and the Spanish version of the health assessment questionnaire disability index (HAQ-DI) were applied to the patients. The second group was comprised of 100 healthy subjects, referred to as control subjects (CS), recruited from the general population. Both groups were unrelated individuals from the same population, and to prevent population heterogeneity, only Mestizo subjects from Southern Mexico were included (specifically from Guerrero state) with at least back three generations of Mexican ancestry.

Quantification of autoantibodies and MIF serum levels

Antibodies against topoisomerase 1 (anti-Scl70, BioSystems Cat. No. COD44863), anti-centromere (CENP-B, BioSystems Cat. No. COD44865), anti-fibrillarin (AFA/snoRNP/U3RNP, CUSABIO Cat. No. CSB-E09697h), and anti-RNA polymerase III (anti-RNA PoIII, CUSABIO Cat. No. CSB-EQ027833HU) were measured with a second-generation enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendations. The sensitivity tests were 0.5 U/mL (anti-Scl70) and 0.3 U/mL (CENP-B). For calculation of the valence AFA and anti-RNA PoIII, we compare the sample well with control (ratio: OD-sample/OD-negative control). Subjects with OD values ≥2.1 were regarded as AFA and anti-RNA PoIII positive. Samples were also tested for anti-nuclear antibody (ANA) by indirect IF microscopy using multisport slides with fixed HEp-2 cells (BioSystems, Barcelona, Spain) according to the manufacturer's recommendations.

MIF serum levels from all individuals were determined by the ELISA method using the Human MIF ELISA Kit Protocol (LEGEND MAX Human Active MIF ELISA Kit, BioLegend) according to the manufacturer's instructions. The detection limit was 17.4 ± 9.2 pg/mL.

Multiplex serum cytokine immunoassay

The serum levels of Th1 (IFN-γ and TNF-α), Th2 (IL-4 and IL-10), and Th17 (IL-17A, IL-17F, IL-1β, IL-6, IL-21, and IL-23) cytokine profile were measured using the 15-Plex #

171-AA001M assay (Bio-Plex® MAGPIX™ Bio-Rad), with a range assay sensibility of 0.2–0.8 pg/mL; those samples with cytokine concentrations below the lowest point on the standard curve were reported with the lowest value. Serum samples were stored at − 80 °C until the day of the assay and then thawed and processed according to the manufacturer's instructions. To corroborate our results, the TNF-α cytokine (LEGEND MAX Human Active TNF-α ELISA Kit, BioLegend) was measured additionally in some samples with a conventional ELISA kit. The TNF-α assay sensitivity was 1.7 pg/mL. The cytokine values obtained with the ELISA kit were correlated highly with this bead-based assay ($r = 0.91$, $p < 0.001$).

Genotyping of − 794 $CATT_{5–8}$ and − 173G>C MIF polymorphisms

Total genomic DNA (gDNA) was isolated from peripheral blood leukocytes by the salting out method [21]. The − 794 $CATT_{5–8}$ *MIF* polymorphism was analyzed by conventional polymerase chain reaction (PCR) and polyacrylamide gel electrophoresis.

The PCR was performed using the primers reported by Radstake et al. [22]. Cycling conditions were initial denaturing 95 °C for 4 min, followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C; and then a final extension of 2 min at 72 °C. Amplification products were further electrophoresed on a 19:1 (7%) polyacrylamide gel at 120 V during 3 h and stained with 0.2% $AgNO_3$ [10, 12].

The − 173G>C *MIF* polymorphism was genotyped by the PCR-RFLP (restriction fragment length polymorphism) technique. Amplification of the polymorphic fragment was done using the primers reported by Makhija et al. [23]; initial denaturing 95 °C for 4 min followed by 33 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C; and then a final extension of 2 min at 72 °C. The 366-bp fragment obtained was further digested with the *Alu I* restriction endonuclease (New England Biolabs, Ipswich, MA, USA) by overnight incubation at 37 °C. Finally, the digestion was resolved on a 29:1 (6%) polyacrylamide gel stained with 0.2% $AgNO_3$. The − 173*G allele resulted in fragments of 268 bp and a 98 bp, while the 173*C allele was represented by 206-bp, 98-bp, and 62-bp fragments [10, 12].

To confirm the results, genotyping of both polymorphisms was done in duplicate in all cases and confirmed by automatized sequencing of a randomly selected subset of − 794 $CATT_{5–8}$ and − 173G>C *MIF genotypes* (Applied Biosystems, USA).

MIF mRNA expression analysis

The peripheral blood sample was collected in EDTA blood collection tubes (BD Vacutainer NJ, USA). The total leukocyte was isolated using dextran reagent (5%) (Sigma-Aldrich Co.), and the total RNA was obtained using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) conforming to the Chomczynski and Sacchi method [24]. The RNA concentration and purity were measured by spectrophotometry (ratio A260/A280) (NanoDrop 2000, Thermo Scientific). After, the cDNA was synthesized from 1 μg of total RNA, and the reverse transcription was performed using primer oligodT (Promega Corporation, USA) as indicated by the manufacturer.

We conducted the quantification of *MIF* mRNA by real-time PCR, using UPL hydrolysis probes (Roche Applied Science, Penzberg, Germany). The primers and probes for quantification were obtained with a design program by Roche Applied Science (Universal Probe Library Assay Design Center), using the sequence of *MIF* mRNA with the NCBI ID number NM_002415.1 (40 number test, Cat. No. 04687990001); the following nucleotide sequences were used as primers: 5′-ACCGCTCCACAGCA AGC-3′ (forward) and 5′-CGCGTTCATGTCGTAATAGT TG-3′ (reverse). The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene (Cat. No. 05190541001). The PCR reaction was performed on a LightCycler Nano System (Roche Applied Science, Germany). All samples were run in triplicate using the conditions indicated in the UPL Gene Expression Assay protocol in a LightCycler Nano System (Roche Applied Science).

After validation of the reaction efficiency for the target gene (*MIF*) and the reference gene (*GAPDH*), the mRNA expression analysis was performed through $2^{-\Delta\Delta Cq}$ and $2^{-\Delta Cq}$ methods. Despite that both two methods are comparable, we chose to use both methods because the $2^{-\Delta\Delta Cq}$ method reports only the global (per group) relative changes in gene expression, whereas $2^{-\Delta Cq}$ method reports individual expression values for each patient, so statistical tests can be used to determine differences between the study groups [25].

Statistical analysis

Categorical variables were expressed as percentages and absolute frequency. The distributions of all continuous variables were examined using the Shapiro–Wilk normality test. Continuous variables distributed normally were expressed as the mean ± standard deviation (S.D.), and those non-normally distributed were expressed as median and 5–95th centiles. Mann–Whitney *U* test was used to evaluate differences between two groups. Kruskal–Wallis test was used to analyze differences between three or more groups (for variables distributed non-normally) followed by Dunn's adjustment for multiple comparisons. Linear correlation coefficients were examined using the Spearman's correlation test. For the

genetic analysis, the Hardy-Weinberg equilibrium and comparisons of allele and genotype distributions between groups were evaluated with the χ^2 or Fisher exact test, as appropriate. Haplotypes were reconstructed using the SHEsis software [26]. The odds ratio (OR) and 95% confidence interval (95% CI) were estimated to analyze the risk of *MIF* genotypes and haplotypes associated with SSc. Statistical analysis was performed using STATA version 11.1 and GraphPad Prism version 6.0 Software. A probability (p) value of less than 0.05 was considered significant.

In order to assess the robustness of the results, the power of each statistical test was conducted according to the suggestion by Hong and Park [27] for genetic association studies. This analysis was performed using the G power 3.1. program [28] and the web browser program "Genetic Power Calculator" [29].

Results

Clinical and demographic characteristics

The study group consisted of 50 patients diagnosed with SSc (43 females and 7 males; aged 48 ± 15.5 years), and the control group consisted of 100 subjects matched for gender and age with the SSc group. The most common type of systemic sclerosis was lcSSc (86%), the median of the evolution of the disease was 5.5 years, and the average age of onset of this disease was 40 years. The ANAs (78%) and CENP-B (28%) were the most frequent autoantibodies in the patients. At the time of inclusion, 62% of the patients were being treated with anti-rheumatic drugs modifying the disease (DMARD), mainly methotrexate (46%), and 39% of the patients did not have treatment. The demographic and clinical characteristics of the SSc patients are shown in Table 1.

Table 1 Clinical and demographic features of SSc patients

Characteristics	SSc ($n = 50$)
Demographics	
Age (years[a])	48.26 (± 15.5)
Female[b]	86 (43)
BMI (kg/m^2)[c]	24 (± 3.9)
Exposure to synthetic fertilizer[b]	40 (20)
Exposure to wood smoke[b]	82 (41)
Clinical assessment	
dcSSc[b]	86 (43)
lcSSc[b]	14 (7)
Age at disease onset (years[a])	40 (± 16)
Disease duration (years[c])	5.5 (0.6–21)
Raynaud's phenomenon[b]	76 (38)
Skin thickening[b]	90 (45)
Telangiectasia[b]	60 (30)
Digital ulcers[b]	42 (21)
Puffy fingers[b]	72 (36)
Joint contractures[b]	86 (43)
Mouth opening (cm)[c]	5 (3–7)
mRSS[c]	6 (0–22)
Spanish HAQ-DI total score (0–3[c])	0.29 (0–1.29)
Autoantibodies[b]	
ANA	78 (39)
CENP-B	28 (14)
anti–Scl70	6 (3)
anti–RNA PoIII	2 (1)
AFA	12 (6)
Drug treatment[b]	
None	38 (19)
Prednisone	36 (18)
Methotrexate	46 (23)
Chloroquine	14 (7)

SSc systemic sclerosis, *BMI* body mass index, *dcSSc* diffuse cutaneous systemic sclerosis, *lcSSc* limited cutaneous systemic sclerosis, *mRSS* modified Rodnan skin score, *Spanish HAQ-DI* The Spanish version of health assessment questionnaire disability index, *ANA* antinuclear antibodies, *CENP-B* antibodies against centromere, *anti-Scl70* antibodies against topoisomerase 1, *anti-RNA PoIII* antibodies against RNA polymerase III, *AFA* antibodies against fibrillarin

[a] Data provided in mean ± standard deviation

[b] Data provided in percentage and n

[c] Data provided in median (p5–p95)

Distribution of the MIF − 794 $CATT_{5–8}$ and − 173G>C polymorphisms and haplotype analysis

The distribution of the genotypic and allelic frequencies of the polymorphisms − 794 $CATT_{5–8}$ and − 173G>C of the *MIF* gene in SSc patients and CS is summarized in Table 2. The two polymorphisms of the *MIF* gene were in Hardy-Weinberg equilibrium in the CS group (− 794 $CATT_{5–8}$ *MIF*, $\chi^2 = 4.5$, $p = 0.55$; − 173G>C *MIF*, $\chi^2 = 2.4$, $p = 0.14$). We found significant differences in the genotypic and allelic frequencies between SSc patients and CS for each of the two polymorphisms evaluated. Mainly, allele 7 of STR − 794 $CATT_{5–8}$ (OR 2.09, 95% CI 1.16–3.77, $p = 0.01$) and allele C of − 173G>C SNP (OR 2.00, 95% CI 1.17–3.43, $p = 0.01$) were associated with an increased SSc risk in our study population.

In addition, a strong linkage disequilibrium was identified between both two *MIF* gene polymorphisms (D' value = 0.89, $r^2 = 0.489$, $p < 0.001$, Fig. 1). Based on the finding of a strong LD between the two studied polymorphisms, we performed a haplotype analysis in patients with SSc and CS (Table 2). Five different haplotypes were identified in our population, where the G6 (− 173G/− 794 $CATT_6$) and C7 haplotypes (− 173C/− 794 $CATT_7$) were the most frequent, representing 81% and

Table 2 Genotype, allele and haplotype frequencies of − 794 $CATT_{5–8}$ and − 173G>C *MIF* polymorphisms in SSc and control subjects

Polymorphism	SSc $n = 50$, % (n)	CS $n = 100$, % (n)	OR (CI 95%)	p
MIF − 794 $CATT_{5–8}$				
Genotype				
5,5	0 (0)	3 (3)	–	–
5,6	16 (8)	21 (21)	1.57 (0.45–5.34)	0.47
5,7	18 (9)	8 (8)	4.63 (1.18–18.06)	< 0.01
6,6[a]	28 (9)	37 (37)	1	–
6,7	36 (18)	21 (21)	3.52 (1.22–10.48)	0.01
7,7	12 (6)	10 (10)	2.47 (0.57–10.02)	0.15
Alelle				
5	17 (17)	17.5 (35)	1.28 (0.61–2.63)	0.47
6[a]	44 (44)	58 (116)	1	–
7	39 (39)	24.5 (49)	2.10 (1.17–3.75)	< 0.01
MIF − 173G>C				
Genotype				
GG[a]	32 (16)	55 (55)	1	–
GC	52 (26)	34 (34)	2.63 (1.16–6.02)	0.01
CC	16 (8)	11 (11)	2.5 (0.73–8.17)	0.09
Alelle				
G[a]	58 (58)	72 (144)	1	–
C	42 (42)	28 (56)	1.86 (1.09–3.17)	0.01
Haplotype				
6G[a]	42 (42)	56 (112)	1	–
5C	1 (1)	1 (2)	1.37 (0.02–26.83)	0.78
5G	16 (16)	16 (32)	1.33 (0.62–2.81)	0.42
6C	2 (2)	2 (4)	1.33 (0.12–9.68)	0.74
7C	39 (39)	25 (50)	2.08 (1.17–3.73)	< 0.01

Chi-square test χ^2. For both two SNPs, the power to detect an OR among 1.86–4.63 under a co-dominant genetic model was of 58–70%. This analysis was done using the software "Genetic Power Calculator," considering the following assumptions: 39% high-risk allele frequency, 0.02% disease prevalence in the Mexican population, linkage disequilibrium of $D' = 89$, 1:2 case-control ratio, and 5% type I error rates

SSc systemic sclerosis, *CS* control subjects, *OR* odds ratio, *CI* confidence interval

[a] Reference category

80% in SSc patients and CS, respectively. The C7 haplotype was found more frequently in patients with SSc compared to CS, confirming its association as a susceptible haplotype in our population (OR 2.08, 95% CI 1.17–3.7, $p < 0.01$) (Table 2). The demographic and clinical characteristics were not associated with the genotypes or haplotypes of *MIF* (− 794 $CATT_{5–8}$ and − 173G>C) (data not shown).

MIF mRNA expression

MIF mRNA expression was evaluated in SSc patients and CS (Fig. 2a, b). This analysis by the $2^{-\Delta\Delta Cq}$ method showed that *MIF* mRNA expression in SSc was 2.7-fold less compared to CS; this difference was significant when assessed by the $2^{-\Delta Cq}$ method ($p < 0.01$).

To investigate the impact of the *MIF* polymorphisms on the mRNA expression, we only considered homozygosity haplotypes (HH), which are haplotypes, formed by only homozygous patients for each SNPs. The *MIF* mRNA expression according to the susceptibility (7C/7C) and non-susceptibility (6G/6G) haplotypes was evaluated in SSc patients and CS. In CS, *MIF* mRNA expression was similar in carriers of both haplotypes ($p = 0.86$; Fig. 2c, d). However, in SSc patients, we found that carriers of the 7C/7C haplotype had 2.3-folds more *MIF* expression than carriers of the 6G/6G haplotype (Fig. 2e, f, $p = 0.03$).

Analysis of MIF serum levels according to MIF promoter polymorphisms

MIF serum levels were compared in SSc patients and CS, but we did not find significant differences between both groups ($p = 0.51$, data not shown). To determine whether the *MIF* promoter polymorphisms were associated with serum protein

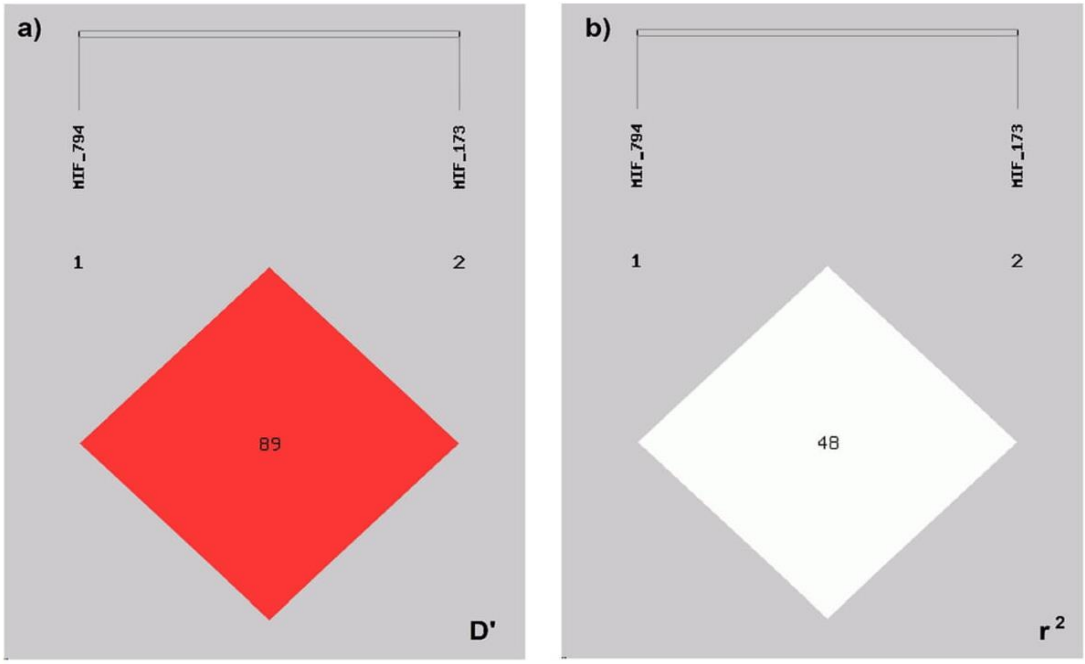

Fig. 1 Pairwise linkage disequilibrium relationships between the MIF variants. **a**) The Lewontin's coefficient D' and **b**) the correlation coefficient r^2 were calculated using SHEsis software. Higher values and darker squares indicate stronger linkage disequilibrium between the polymorphisms (*MIF* − 794 $CATT_{5–8}$ and *MIF* − 173G>C)

levels, the genotypes were grouped according to the dominant genetic model proposed for each polymorphism in both study groups. Nevertheless, the genotypes of two polymorphisms of the *MIF* gene did not show an association with MIF levels. MIF serum levels were also tested for association with *MIF* haplotypes, but did not find significant differences in SSc patients ($p = 0.54$, data not shown).

Serum levels of Th1/Th2/Th17 cytokines

The comparison between serum levels of Th1/Th2/Th17 cytokines in SSc patients and CS is shown in Table 3. In the Th1 cytokine profile (IFN-γ and TNF-α) did not observe significant differences between SSc patients and controls ($p = 0.137$ and $p = 0.372$, respectively). Concerning the Th2 cytokine profile (IL-4 and IL-10) also did not find significant differences when comparing both study groups ($p = 0.212$ and $p = 0.733$, respectively). Nevertheless, differences between both study groups were found concerning Th17 cytokines levels: The serum levels of IL-17A show a median of 5.91 pg/mL in CS vs. 3.87 pg/mL in SSc patients ($p = 0.013$), whereas for IL-1β, the median observed was 0.65 pg/mL in SSc patients versus 0.24 pg/mL in CS ($p = 0.025$); similarly, significant differences were identified for IL-6 cytokine between both study groups (SSc median 20.39 pg/mL vs. CS median 9.33 pg/mL, $p < 0.01$).

Additionally, the correlation of the Th1/Th2 and Th17 cytokines profiles with MIF serum levels in SSc patients and CS was analyzed (Table 4). In the SSc group, we found positive correlations of MIF with the IFN-γ ($r = 0.63$; $p = 0.04$) and TNF-α ($r = 0.69$; $p < 0.01$) cytokines of Th1 profile, but in the CS, this correlation was not observed. Regarding the Th2 profile (IL-4 and IL-10), we did not observe any correlations with MIF in both study groups. Meanwhile in the Th17 profile, a positive correlation of MIF with IL-17A ($r = 0.59$; $p = 0.02$), IL17F ($r = 0.66$; $p < 0.01$), IL-1β ($r = 0.68$; $p < 0.01$), and IL-21 ($r = 0.51$; $p = 0.01$) was found only in the patient's group.

Discussion

SSc is an autoimmune, inflammatory, chronic, and multifactorial disease of unknown etiology [1], but it can be influenced by environmental and genetic factors [6]. Within the genetic factors, several genetic polymorphisms involved in SSc susceptibility and severity have been described in the *HLA-DR* locus. Other associated genes comprise *IRF5* (rs607218), *STAT4* (rs600558), *IL12RB2* (rs601642), *PXK* (rs611450), and *MIF* (rs755622) [17, 18, 30].

Two independent studies observed that the *MIF* − 173G<C polymorphism was strongly associated with dcSSc [17, 18],

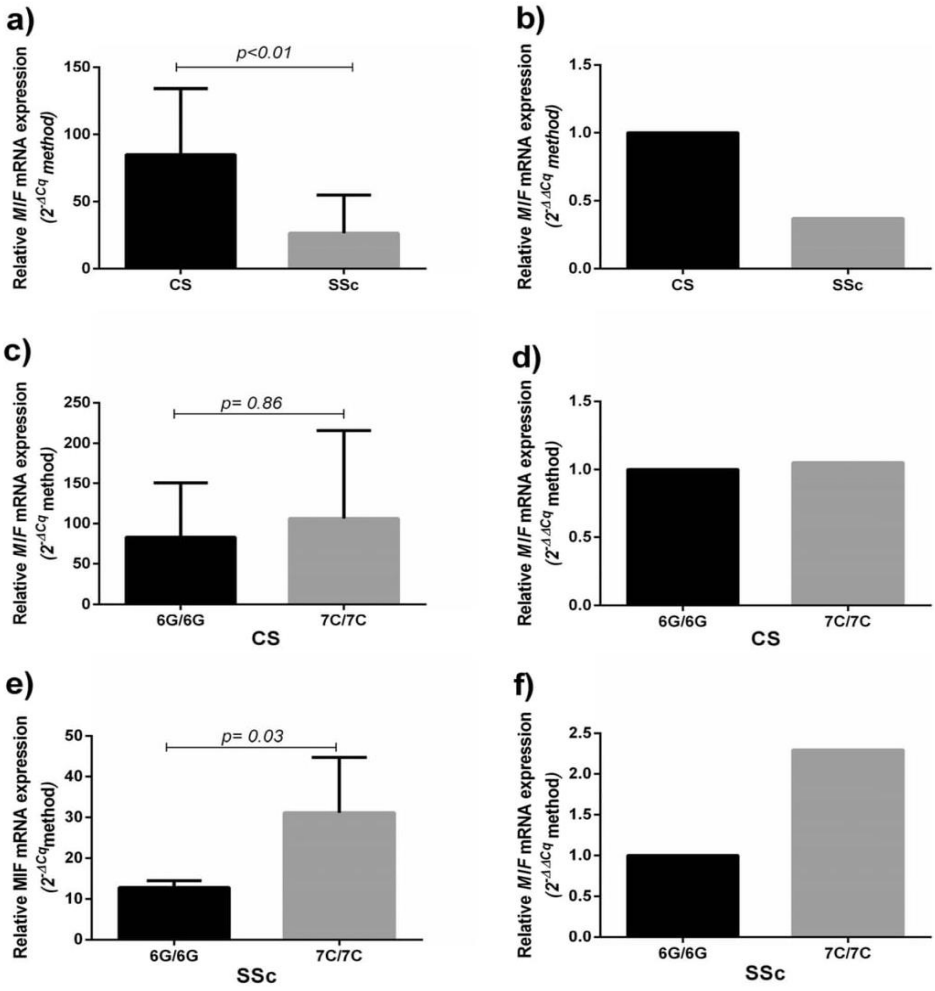

Fig. 2 Relative expression of mRNA. *MIF* mRNA expression in CS and SSc (**a**, **b**). *MIF* mRNA expression in susceptibility (7C/7C) and nonsusceptibility (6G/6G) haplotype carriers. The analysis was performed in CS (**c**, **d**) and SSc (**e**, **f**). Relative expression analysis was performed using $2^{-\Delta Cq}$ and $2^{-\Delta\Delta Cq}$ methods, and *GAPDH* was the reference gene. Statistical comparisons between groups were made using the Mann-Whitney *U* test

and this polymorphism has been also associated with a high production of MIF in other autoimmune diseases such as RA [22] and SLE [31]. In our study, we identified an association between *MIF* genetic variants (− 794 $CATT_7$ and − 173*C) and its haplotype C7 with SSc, so we suggest that these polymorphisms may confer susceptibility to develop of SSc. However, this result should be interpreted with caution due to the moderate (58–70%) statistical power observed [32]; this does not reduce the reliability of the association obtained, but it is known that the effect size can be overestimated when the power is moderate or low [33].

These results are according to those reported in the North American and Caucasian (eight European populations) populations, where they have confirmed not only an association between the − 173*C *MIF* allele with SSc risk, but they have been observed a greater frequency of C allele in the dcSSc subgroup of patients [17, 18]. One of the limitations of our study for this analysis was that we did not stratify the SSc into localized and diffuse types since the latter had a very low frequency (14%).

Regarding SSc, the MIF − 794 $CATT_{5-8}$ polymorphism has been evaluated only by Wu et al., in the North American population (The United States of America and Canada), but unlike our population, they did not observe an association between the polymorphism and SSc [17]. This discrepancy could be explained by the different genetic structure that exists between both populations, as the population evaluated by Wu et al. was white population from The United States of America and Canada, which ancestry could be mainly European [34, 35], while the ancestry of our study population is predominantly Amerindian (48%), followed by Europe (38%), Asian (10%), and African (4%) [36]. Therefore, there is a need to further investigate the impact of genetic variations at the *MIF* gene in additional populations, with greater sample sizes, and with a better definition of clinical phenotypes.

Table 3 Cytokine serum levels in SSc patients and CS

Cytokines (pg/mL)	SSc ($n = 50$)	CS ($n = 50$)	p
Th1 profile			
IFN-γ	17.97 (3.69–217.68)	9.49 (3.69–26.45)	0.137
TNF-α	3.33 (0.73–9.45)	3.26 (0.87–7.08)	0.372
Th2 profile			
IL-4	18.41 (1.68–62.08)	25.14 (6.24–64.43)	0.212
IL-10	5.36 (1.27–26.43)	4.59 (1.27–11.77)	0.733
Th17 profile			
IL-17A	3.87 (1.32–7.94)	5.91 (0.8–21.31)	0.013
IL-17F	25.79 (2.32–106.2)	20.92 (10.9–46.81)	0.677
IL-1β	0.65 (0.05–2.21)	0.24 (0.02–0.46)	0.025
IL-6	20.39 (8.93–63.29)	9.33 (4.3–20.79)	< 0.01
IL-21	38.33 (9.63–689.01)	25.36 (6.33–95.52)	0.057
IL-23	278.31 (20.73–962.5)	166.90 (90.28–278.31)	0.470

Data provided in median (p5–p95). The p values were calculated Mann-Whitney test. All significant test showed a statistical power > 0.90, according to G power 3.1 program

SSc systemic sclerosis, *CS* control subjects, *MIF* macrophage migration inhibitory factor, *IFN-γ* interferón gamma, *IL* interleukin, *TNF-α* tumor necrosis factor alpha

In RA, it has been observed that carriers of the − 173*C and − 794 $CATT_7$ *MIF* alleles have a high activity of the disease classified by a DAS28 score in comparison with those patients carrying another allele [10, 14, 22]. Our research group have found several genetic associations of these *MIF* polymorphisms with susceptibility to RA [10], SLE [12], PsA [11], and MS [13] in population from western Mexico, which provides evidence that *MIF* gene could be a susceptibility biomarker of autoimmunity in the Mexican population. In the present study, the association of *MIF* polymorphisms with clinical evaluation indices, clinical manifestations, and autoantibodies in SSc patients was investigated, but we did not observe significant differences. In this regard, the previous similar studies only have investigated the association of *MIF* polymorphisms with anti-Scl70 and CENP-B autoantibodies without significant differences observed [17, 18]; for this reason, we cannot rule out such associations.

A striking observation in our study was a higher *MIF* mRNA expression in CS than in SSc patients. It is interesting to note that this is the first study reporting *MIF* mRNA expression in peripheral leukocytes from SSc patients. However, Corallo et al. showed that *MIF* mRNA expression is higher in cultures of fibroblasts from SSc patients than those from CS [37]. A possible explanation for this finding might be that in SSc patients, the production of MIF mRNA can be higher in skin cells than in leukocytes. It is possible, therefore, that MIF may have an important role in the localized inflammatory process (the skin) in SSc patients but not at the systemic level. Also, this finding could be also explained by the treatment of the SSc patients, since it has been reported that some antirheumatic drugs, such as chloroquine, can negatively regulate the mRNA expression of proinflammatory cytokines (TNF-α and IL-1β) that correlate positively with the expression of MIF [38]. Thus, it is possible that this kind of drugs downregulated the MIF expression; however, this hypothesis should be tested in a larger cohort.

Another important finding was that patients with the susceptibility *MIF* haplotype (7C) had higher expression of *MIF* mRNA in comparison with carriers of other haplotypes. These changes in expression could be due to differences in the transcription factor interactions with the two polymorphisms of the *MIF* promoter that were studied. In the case of STR − 794 $CATT_{5–8}$, the transcription factor ICBP90 binds to these repetitions, regulating positively the expression of this gene [15].

We did not find significant differences between MIF serum levels in both study groups; unlike other studies, it has been observed increased MIF serum levels in patients with SSc [5, 9, 17, 19]. We suggest that this discrepancy may be due to various factors, such as the significant differences observed in BMI (body mass index) between both groups ($p = 0.02$, data not shown). Although our CS does not attend to obesity, the average of their BMI places them in overweight (28 kg/m^2, data not shown), while the SSc patients were placed in normal weight (24 kg/m^2). Excess weight and obesity are characterized by a state of chronic low-level systemic inflammation [39, 40], which is caused by the expansion of adipose tissue. It is accompanied by a progressive infiltration of leukocytes in the adipose tissue (AT), which can be attributed to hypersecretion by the AT of proinflammatory cytokines, such as MIF, IL-6, TNF-β, IL-1β, and MCP-1 [41, 42]. In addition, there are more factors that can modulate MIF such as levels of antidiabetic drugs [43], glucocorticoids [44], or even drastic weight loss [45]. On the

Table 4 Correlation of MIF serum levels with Th1, Th2, and Th17 cytokine profile of SSc patients and CS

Cytokines	MIF			
	SSc		CS	
	r	*p*	*r*	*p*
Th1 profile				
IFN-γ	0.63	0.04	0.36	0.15
TNF-α	0.69	< 0.01	− 0.20	0.24
Th2 profile				
IL-4	0.03	0.90	− 0.18	0.50
IL-10	0.12	0.49	0.02	0.92
Th17 profile				
IL-17A	0.59	0.02	− 0.32	0.09
IL-17F	0.66	< 0.01	− 0.07	0.86
IL-1β	0.68	< 0.01	0.09	0.83
IL-6	0.21	0.16	− 0.12	0.48
IL-21	0.51	0.01	− 0.04	0.87
IL-23	0.11	0.74	− 0.06	0.76

The *p* and *r* values were calculated by Spearman's correlation test. All significant test showed a statistical power > 0.90, according to G power 3.1 program

SSc systemic sclerosis, *CS* control subjects, *MIF* macrophage migration inhibitory factor, *IFN-γ* interferon gamma, *IL* interleukin, *TNF-α* tumor necrosis factor alpha

other hand, the Toll-like receptor 4 (TLR4), induces a release of MIF through the signal transduction of the LPS receptor complex; however, a recent study has shown that the long-term use of chloroquine decreased both the expression of mRNA and TLR-4 proteins in monocytes [38, 46–48]. In this way, it could indirectly affect the MIF levels.

Regarding the cytokines involved in the pathogenesis of SSc, the participation of cytokines from the Th2 and Th17 profiles has been observed, among which TGFβ, IL-6, IL-1β, IL-21, IL-10, IL-13, among others are the most associated [49–52]. It has been reported higher levels of IL-10, TNFα, and Th17 cytokine profile in serum and exhaled breath condensate (EBC) from patients with dcSSc and lcSSc than in controls. Furthermore, the cytokines are higher in dcSSc than in lcSSc [53]. In accordance with those results, we observed high levels of Th17 cytokine profile members (IL-1β and IL-6) in the SSc group, but we did not find differences between serum levels of all cytokines evaluated according to the type of SSc.

The increased levels of the Th17 cytokine members may explain the relatively good correlation between Th17/Th1 cells and some adhesion molecules such as L-selectin and ICAM, which are overexpressed in SSc, and both regulate the accumulation of Th2 and Th17 cells in the skin and lung, which leads to the development of fibrosis. On the other hand, other adhesion molecules such as P-selectin, E-selectin, and PSGL-1 regulate the infiltration of Th1 cells, which results in the inhibition of fibrosis [50].

Controversially, in our study, we did not find significant differences between SSc patients and controls in the Th2 cytokine profile, where IL-10 and IL-4 were measured. However, several studies have consistently described the involvement of Th2 cells in the pathogenesis of SSc and elevated levels of their cytokines such as IL-4 and IL-13. It is known that IL-4 is critical for the polarization of Th2 cells; however, IL-13 is also necessary to mount an appropriate Th2 response in SSc [52]. Therefore, we suggest that it would be important to measure other cytokines of the Th2 profile, such as IL-13 in the future, to corroborate our results. In addition, we investigated whether there were differences between the patients with and without treatment, and curiously, we observed that the levels of IL-4 are diminished in the group of patients with treatment ($p = 0.02$; data not shown). Due to the above, we suggest that the treatment could be affecting the levels of IL-4, based on a recent study that states that drugs such as glucocorticoids (dexamethasone) synergy with biological drugs are directed to IL-4 (F8-IL4) in the treatment of chronic inflammatory conditions [54].

Recently, it has been suggested that MIF is a cytokine with profibrotic functions, as an inhibitor of the apoptosis of the fibroblasts and playing an important role in the stimulation of fibroblasts for the production of collagen and extracellular matrix in SSc [9, 20]. In the same way, MIF is associated with immunomodulation of Th1, Th2, and Th17 cytokine profile in inflammatory diseases. A positive correlation of MIF with cytokine TGF-β has also been observed in SSc [9]. In our study, we observed a positive correlation between the MIF serum levels and cytokines of the Th1 (IFN-γ and TNF-α) and Th17 (IL-17A, IL-17F, IL-1β, and IL-21) profile in SSc patients but not in control subjects. Although to date there is only a report of the correlation between MIF and TGF-β in SSc [9], our research group has evaluated this correlation in other autoimmune diseases, such as PsA [55] and SLE [56]. In PsA, the correlation between MIF and TNF-α expression and the Th1, Th2, and Th17 cytokine profile was evaluated; we observed that high expression of TNF-α mRNA increases the profiles of Th1 cytokines (IFN-γ and TNF-α) and Th17 (IL-17 e IL-22), but this correlation was not observed with the MIF expression [55]. In PBMCs of SLE and CS, it was observed that MIF induces the inflammatory response in both physiological (CS) and pathological (SLE) conditions with a predominance of the Th17 profile in CS and an increase in TNF-α and IL-6 in active SLE. This suggests that MIF is an immunomodulatory cytokine that might not have predominance towards a specific Th profile [56].

In conclusion, this study provides evidence that the individual polymorphisms and functional haplotype in *MIF* are associated with susceptibility to SSc and high *MIF* mRNA expression in a Mexican-Mestizo population from southern

Mexico. In the same way, the results obtained suggest that MIF is associated with a proinflammatory response in SSc, as it correlates positively with the Th1 (IFN-γ and TNF-α) and Th17 (IL-17A, IL-17F, IL-1β, and IL-21) cytokine profile. Further research should be undertaken to evaluate these cytokine profiles in cultures of PBMCs or skin cells to have more forceful results.

Funding information The study was supported by funding from the National Council of Science and Technology (CONACYT) Grant A1-S-8774 (CONACYT Ciencia Básica-Universidad de Guadalajara) assigned to JFMV. Also, CJH-B was a Ph.D. fellow at CONACYT-Mexico (#301502).

Compliance with ethical standards

Informed written consent was obtained from all subjects before enrollment to the study. The investigation was performed according to the ethical guidelines of the 2013 Declaration of Helsinki and was approved by the ethical, investigation, and biosecurity committee of the Hospital General de Chilpancingo "Dr. Raymundo Abarca Alarcón," Chilpancingo de los Bravo, State of Guerrero, Mexico (CI/317/2016).

Disclosures None.

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CAPÍTULO II

"*Expression of macrophage migration inhibitory factor (MIF) and its receptor CD74 in systemic sclerosis*"

Expression of macrophage migration inhibitory factor (MIF) and its receptor CD74 in systemic sclerosis

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Abstract

Background: The macrophage migration inhibitory factor (MIF) has been studied in some rheumatic diseases, such as systemic sclerosis (SSc), where it has shown that MIF expression is dysregulated in serum and skin culture derived supernatant. CD74 is a type II transmembrane glycoprotein and a MIF receptor, by which functions such as proliferation and cell survival are carried out, however, its potential role in the pathogenesis of SSc remains poorly understood.

Objective: To analyze the mRNA, tissue, and serum expression of MIF and CD74 in patients with limited (lcSSc) and diffuse (dcSSc) systemic sclerosis.

Subjects and methods: A case-control study in 20 SSc patients and 20 control subjects (CS) from southern México was carried. The *MIF* and *CD74* mRNA expression were quantified by real-time PCR, MIF serum levels were measured by ELISA kit, and MIF and its receptor CD74 were evaluated by immunohistochemistry on skin biopsies.

Results: *MIF* mRNA expression was significantly higher in CS than in SSc patients (p=0.02); while *CD74* showed no differences between patients and CS. MIF serum levels were similar between SSc patients and CS: dSSc = 3.82 ng/mL, lSSc = 3.57 ng/mL, and CS (3.28 ng/mL). In skin biopsies of SSc, the immunostaining of MIF and CD74 were enhanced in keratinocytes, while they decreased expression in endothelial cells. On the other hand, the staining of CD74 was high in fibroblasts of dcSSc patients.

Conclusions: Our findings show a MIF and CD74 deregulation at the transcriptional and translational levels in SSc, which might be associated with the proinflammatory process that leads the tissue remodeling and excessive fibrosis in SSc.

Keywords: Sclerosis systemic, MIF, CD74, Skin, Immunohistochemistry.

Introduction

Systemic sclerosis (SSc), also called scleroderma, is a multisystem autoimmune disease characterized by immune system activation, vascular injury, and tissue fibrosis of the skin and certain internal organs [1]. This disease can be classified into two disease subset based on the extent of skin involvement: diffuse cutaneous systemic sclerosis (dcSSc) and limited cutaneous systemic sclerosis (lcSSc) [2]. The prevalence of SSc in the USA has been calculated in approximately 250 persons per million inhabitants[3] ;in the Mexican population, no accurate data of prevalence and incidence has been reported for SSc, however, the overall prevalence of scleroderma has been reported at 0.02% [4]. The triggering agents of SSc to date is unknown, however, it has been considered a multifactorial disease where contribute genetic, infectious and environmental factors [5].

Emerging evidence suggests that the macrophage migration inhibitory factor (MIF), a pleiotropic inflammatory cytokine with a broad range of immunomodulatory properties, could play a role in the pathogenesis of SSc, but the exact mechanism is unclear. In 2003, Selvi et al. reported that serum concentrations of MIF were significantly higher in dcSSc patients than in controls, and MIF expression was detected in skin biopsies of SSc patients by immunohistochemical staining [6]. In addition, Becker et al. revealed that MIF can contribute to vasculopathy in the SSc [7]. On the other hand, it was reported that MIF stimulates the process of excessive fibrosis in SSc, increasing the proliferation of fibroblasts and collagen synthesis [8], and decreasing the apoptosis of dermal fibroblasts [9].

CD74 is a molecule associated with the processing of MHC class II proteins and it is a high-affinity cell membrane receptor for MIF (~ 9 × 10-9 Kd) [10]. A small proportion of CD74 is modified by the addition of chondroitin sulfate (CD74-CS), and this form of CD74 is expressed on the cell

surface [11]. However, MIF signaling through CD74 depends on the interaction with co-receptors, like CD44, which through their cytoplasmic domains are able to recruit the necessary components to activate signaling [12, 13].

Corallo et al. evaluated MIF and its receptors CD74/CD44 by immunohistochemistry (IHC) on skin biopsies from patients with dcSSc, lcSSc (affected and not-affected skin), and controls and they found enhanced MIF immunoreactivity in keratinocytes, fibroblasts, endothelium, and sebaceous/sweat glands from affected skin of lcSSc/dcSSc patients. Moreover, a faint MIF immunoreactivity was found in control skin and not-affected skin of lcSSc patients; however, no differences were found in CD74/CD44 receptors' analysis among control and dcSSc/lcSSc affected and non-affected skin [14].

Recently, we observed that polymorphisms and functional haplotypes at the *MIF* gene are associated with susceptibility to SSc and high *MIF* mRNA expression in a Mexican-Mestizo population from southern Mexico. In the same way, we found that MIF is associated with a proinflammatory response in SSc, as it correlates positively with the Th1 (IFN-γ and TNF-α) and Th17 (IL-17A, IL-17F, IL-1β, and IL-21) cytokine profiles [15]. Based on our previous results and the existing data, the aim of this study was to analyze whether there was an association between the expression of MIF and its receptor (CD74) at systemic (mRNA and protein) and in situ (skin cells) levels in SSc patients.

Materials and methods

Patients

This study was conducted in 20 SSc patients classified according to the 2013 American College of Rheumatology/European League Against Rheumatism classification criteria for SSc [16]. They were consecutively enrolled from the Rheumatology Department at Hospital General de Chilpancingo "Dr. Raymundo Abarca Alarcón", Chilpancingo de los Bravo, State of Guerrero, Mexico. In the same way, 20 control subjects (CS) recruited from the general population, were included.

Informed written consent was obtained from all subjects before enrollment to the study. The investigation was performed according to the ethical guidelines of the 2013 Declaration of Helsinki and was approved by the ethical, investigation, and biosecurity committee of the Hospital General de Chilpancingo "Dr. Raymundo Abarca Alarcón", Chilpancingo de los Bravo, State of Guerrero, Mexico (CI/317/2016).

MIF and CD74 mRNA expression analysis

The peripheral blood sample was collected in EDTA blood collection tubes (BD Vacutainer NJ, USA). The total leukocyte was isolated using dextran reagent (5%) (Sigma Aldrich Co), and the total RNA was obtained using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) conforming to the Chomczynski and Sacchi method [17]. The RNA concentration and purity were measured by spectrophotometry (ratio A260/A280) (NanoDrop 2000, Thermo Scientific). After, the cDNA was synthesized from 1 μg of total RNA, and the reverse transcription was performed using primer oligodT (Promega Corporation, USA) as indicated by the manufacturer.

We conducted the quantification of *MIF* and *GAPDH* mRNA by real-time PCR, using UPL hydrolysis probes (Cat. No. 05190541001 and 05190541001, Roche Applied Science, Penzberg, Germany). All samples were run in triplicate using the conditions indicated in the UPL Gene Expression Assay protocol in a LightCycler 96 System (Roche Applied Science).

CD74 mRNA expression was quantified using TaqMan probes (Cat. No. 4331182, Applied Biosystems, United States), and the *GAPDH* (Cat. No. 4331182, Applied Biosystems, United States) was used to reference gene. The mRNA expression analysis was performed through $2^{-\Delta\Delta Cq}$ and $2^{-\Delta Cq}$ methods after validation of reaction efficiency for the target genes (*MIF* and *CD74*) and the reference gene (*GAPDH*) [18].

MIF serum levels

The blood samples of all individuals were collected and centrifuged to obtain the sera, which were subsequently stored at −20°C. After, MIF serum levels were determined by the ELISA method using the Human MIF ELISA Kit Protocol (LEGEND MAX Human Active MIF ELISA Kit, BioLegend) according to the manufacturer's instructions. MIF assay sensitivity was 6 pg/mL.

Immunohistochemistry for MIF and CD74

We analyzed the expression of MIF and CD74 in skin biopsy samples. Skin biopsies were obtained at the time of sera collection and were fixed in Michel´s solution for later paraffin embedding. The skin samples were obtained by 4-mm puncture biopsy under local anesthesia from the affected skin. Samples were embedded in paraffin and cut into 5 μm sections for mounting on pre-loaded slides.

Tissues were routinely processed by heat, xylene, and graded ethanol solutions. Once tissue sections were re-hydrated, antigen retrieval was accomplished in a bath of sodium citrate solution 10 mM (pH=6) at 95°C for 10 min followed by cooling in a citrate cold solution. Endogenous peroxidase activity was neutralized

with H_2O_2 (Peroxidase Block) for 10 minutes. For reduce non-specific binding of primary antibody and polymer was applied the Novocastra™ Protein Block. The sections of each biopsy were incubated overnight at 4°C with one of the following primary antibodies: anti-MIF (Cat. ab55445; dilution 1:200) or anti-CD74 LN2 (Cat. ab9514; dilution 1:200). The detection of primary antibodies was performed using the polymer Novolink ™ (Cat: RE7140-CE, Novocastra, UK). Sections were further incubated with the substrate/chromogen, 3,3' -diaminobenzidine (DAB), prepared from DAB Chromogen and Novolink™ DAB Substrate Buffer. Finally, sections were counterstained with hematoxylin.

After staining, images of the slides were captured with a digital camera (Axiocam ICc 1; Zeiss AG, Oberkochen, Germany) attached to an optical microscope (Axio Lab.A1; Zeiss AG). Images were qualitatively analyzed by two pathologists' specialists. The cells that were positive for the brand were quantified according to their lineage and using a positive control as a reference.

Statistical analysis

Data were analyzed as follows: the qualitative variables were expressed as percentages and absolute frequency, and continuous variables distributed normally were expressed as the mean ± standard deviation (s.d.), and those non-normally distributed were expressed as median and 5–95th centiles. Mann–Whitney U-test was used to evaluate differences between the two groups and Kruskal–Wallis test was used to analyze differences between three or more groups (for variables distributed non-normally) followed by Dunn's adjustment for multiple comparisons. Statistical analysis was performed using STATA version 11.1 and GraphPad Prism version 6.0 Software. A probability (p) value of less than 0.05 was considered significant.

Result

Clinical and demographic characteristics of SSc patients and CS

The demographic and clinical data of SSc patients and CS are shown in Table 1. In this study, a total of 20 patients diagnosed with SSc (16 females and 4 males; aged 44.4 ±17.7 years), and 20 CS (15 females and 5 males; aged 39.6 ± 14.6) were included. In SSc patients, a greater percentage of individuals with a classification of lcSSc (86%) compared to dcSSc (14%) was observed. SSc patients had disease evolution of 4 years (0.5-20) at the inclusion time. In the clinical manifestations, most patients had sclerodactyly and joint contractures (95%), followed by puffy fingers (75%), Raynaud's phenomenon (70%) and telangiectasia (50%). Concerning the clinical evaluation, the patients had a mean of HAQ (Health assessment questionnaire disability index) of 0.46 ± 0.44 and a median mRSS51 (Modified Rodnan Skin Score) of 6 (0-30). Most patients were not under treatment (55%) because they were enrolled in the present study at the time of diagnosis by the rheumatologist. On the other hand, patients under treatment were treated mainly with methotrexate (35%) and prednisone (35%), followed by chloroquine (15%).

MIF and CD74 mRNA expression

MIF and *CD74* mRNA expressions were evaluated in SSc patients and CS (Figures 1a,c). According to the $2^{-\Delta\Delta Cq}$ method, *MIF* mRNA expression was 2-fold less in SSc compared to CS (Figure 1a); Similarly, *CD74* mRNA expression was lower (1.4-fold less) in SSc patients compared to CS (Figure 1c). These differences were only significant for the *MIF* mRNA expression when the data were assessed by the $2^{-\Delta Cq}$ method (p=0.02) [19].

To investigate the role of MIF and CD74 according to the types of SSc, we compared the *MIF* (Figure 1b) and *CD74* (Figure 1c) mRNA expression between patients with lcSSc and dcSSc. We

observed that patients with dcSSc had lower *MIF* (1.4-fold) and *CD74* (2.6-fold) mRNA expression than patients with lcSSc, however, this difference was not statistically significant (p>0.05).

Serum levels of MIF according to the type of SSc

We observed a trend of higher MIF serum levels in SSc patients in comparison to the CS group [SSc: 3.72 (0.58-9.5) ng/mL vs CS: 3.28 (0.74-4.37) ng/mL], however, this was not statistically different (p = 0.52, Figure 2a). Subsequently, we compared the MIF serum levels among lcSSc and dcSSc patients, and CS (Figure 2b); in this regard, we observed a trend of higher MIF serum levels in dcSSc patients (3.82 ng/mL), followed by lcSSc patients (3.57 ng/mL) and CS (3.28 ng/mL). However, these differences were not statistically significant (p=0.77).

Detection of MIF and CD74 in skin cells

We analyzed the expression of MIF and CD74 in skin biopsies by IHC. Twenty cases of scleroderma skin were examined and immunostained with anti-MIF and anti-CD74 antibodies. All tissues showed the histological hallmarks of SSc disease, such as the paucity of epidermal appendages and scant cellularity of the reticular dermis, which showed thickened collagen bundles. In the epidermis, we found that both MIF and CD74 were highly expressed in the keratinocytes of SSc patients (Figure 3c,d) followed by the spindle fibroblast-like cells. Likewise, MIF and CD74 were expressed in dermal cells, such as glands and infiltrating mononuclear cells. On the other hand, in endothelial cells both MIF and CD74 were expressed in a lower percentage (Figure 3a,b). In control skin specimens, MIF was strongly expressed in all layers of the epidermis, as well as in endothelium and scarce fibroblasts of the papillary dermis, while, CD74 was irregularly expressed in some keratinocytes of the stratum basale, stratum spinosum, and granulosum; and in endothelial cells (data not shown).

In the stratification of SSc subtypes (lcSSc and dcSSc), CD74 was higher stained in fibroblasts of dcSSc patients (Figure 4b,d) than in lcSSc (Figure 4b,c) (p=0.001). Similarly, MIF showed higher staining in fibroblasts of dcSSc (Figure 4a) patients than in lcSSc patients, however, these differences were not significant (p=0.35).

Discussion

MIF is an immunoregulatory cytokine that inhibits apoptosis induced by p53 activation and regulates the proliferation of different types of cells [20] via its CD74/CD44 receptors [21]. In SSc, it has been suggested that MIF could contribute to skin fibrosis by inhibiting the apoptosis of dermal fibroblasts [9], and it augments fibroblast proliferation and collagen synthesis [8]. However, the participation of MIF receptors in the pathogenesis of SSc has been poorly investigated [14]. To obtain more information about MIF and CD74 role in SSc, we evaluated the MIF serum levels in lcSSc, dcSSc, and CS, followed by the MIF and CD74 mRNA expression in leukocytes and the skin.

Corallo et al. suggest that MIF is produced by fibroblasts at the beginning of their differentiation into myofibroblasts and that MIF probably prevent their apoptosis in SSc [14]; this results described the autocrine effect of MIF in fibroblasts, however, it has also been suggested a paracrine effect of MIF produced by T cells via inhibition of apoptosis in fibroblasts [9]. Therefore, the present study evaluated the *MIF mRNA* expression in peripheral blood leukocytes, where we observe a higher *MIF* mRNA expression in CS than in SSc patients; in this respect, previous studies have been reported that some anti-rheumatic drugs, such as chloroquine, can negatively regulate the mRNA expression of some proinflammatory cytokines (TNF-α and IL-1β) that

correlate positively with the expression of MIF [22]. However, despite a significant percentage of our patients are under treatment, the expression of *MIF* mRNA was not different between SSc patients without or with treatment. To understand the reasons for this discordance, we suggest that the processes involved in the synthesis and degradation of MIF at the tissue and systemic levels must be investigated in future studies in SSc patients.

On the other hand, CD74 has been described as a high-affinity receptor for MIF [23], therefore, we also evaluated the *CD74* mRNA expression in peripheral blood leukocytes. In this regard, higher mRNA expression of *CD74* in CS was observed in comparison with the SSc patients, like that observed in *MIF* mRNA expression. This study is the first to evaluate the *CD74* mRNA expression in SSc patients, therefore, more studies are needed to confirm this finding.

Several studies have shown increased MIF levels in both serum and dermal fibroblasts culture supernatant of SSc patients [7, 9, 24]. In serum, they observed that patients with dcSSc have higher levels of MIF than patients with lcSSc and CS [7, 6]. However, in our population, these significant differences were not observed when comparing the serum levels of MIF between patients with SSc and CS, or when stratifying them by type of SSc. Our results coincide with that reported by Wu et al., who did not observe significant differences of MIF serum levels between types of SSc, however, they argue a tendency to higher levels of MIF in the group of patients with SSc in comparison to the CS group [19]. The discrepancy between the results of the previous studies reflects the variability in the serum levels of MIF in SSc, which can be influenced by the treatment (methotrexate, glucocorticoids, and chloroquine) [25, 26]. On the other hand, it is probable that the serum levels of this protein do not correlate positively with their tissue production (skin, in the case of SSc) [27].

In the present study, the mRNA expression of *MIF* in leukocytes and MIF serum levels showed no correlation. In this regard, it is known that the expression of the mRNA of a particular gene does not always predict the expression of the protein, and the correlation between the two can vary significantly [28]. There are several possible explanations for these differences between mRNA and protein levels, such as post-transcriptional regulation that has shown to play a vital role in controlling the expression of cytokines by modulating mRNA stability [29, 30]. However, it is important to consider the fact that MIF is not only expressed in of the blood cells but is also expressed in skin cells and it is associated with wound repair [31].

In previous studies, it has been documented a crucial role of MIF in the acceleration of wound healing , the response to tissue injury and regulating the immunological and inflammatory phases of the wound process [31, 32]. MIF also shows a chemotactic effect on the keratinocytes of the skin; in addition, fibroblasts of the skin wound produce a greater amount of MIF in response to lipopolysaccharide, and interestingly, this factor is important in the migration of the fibroblasts and the regeneration of the skin after the wound [33, 34]. Taken together, these results could suggest that MIF contributes to the wound healing process of skin tissue, including tissue remodeling and fibrosis, which are characteristic of the SSc.

To obtain more information about MIF and CD74 role in SSc, we examined skin biopsies by IHC. We found that MIF and CD74 were expressed in a higher percentage in keratinocytes followed by fibroblasts, sweat glands, infiltrating mononuclear cells and endothelial cells on the skin. These findings agree to that reported by Selvi, et al., as they observed MIF staining in keratinocytes, fibroblasts, sweat glands, infiltrating mononuclear cells, and endothelial cells. However, they did not evaluate the presence of the CD74 receptor [6].

On the other hand, Corallo et al., also evaluated the presence of MIF and its CD74 receptor in skin biopsies of patients with SSc. They reported an improved immunoreactivity of MIF in keratinocytes, fibroblasts, endothelium, sebaceous/sweat glands of lcSSc/dcSSc patients with skin involvement. They also detected a weak immunoreactivity of MIF in the control subject skin and in the unaffected skin of patients with lcSSc. Whereas, the CD74 / CD44 receptor complex showed no difference between the control and affected and unaffected skin of dcSSc/lcSSc patients [14]. The MIF and CD74 expression in skin cells, mainly in keratinocytes and fibroblasts support the hypothesis that MIF, and probably CD74, plays an important role in the development of fibrotic lesions of the skin in course of SSc. It is known that keratinocytes have an important role in the pathogenesis of SSc, through the activation of fibroblasts independently of TGF-β [35]. Also, it has been described that increased MIF expression may suppress apoptosis in keratinocytes [36].

Finally, we observed that there is a higher percentage of CD74 expression in fibroblasts of dcSSc patients than in lcSSc patients. Contrary to this, Corallo, et al., did not find differences in the analysis of the CD74/CD44 receptors between the control and dcSSc/lcSSc affected and unaffected skin [14]. MIF also seems to have a higher percentage of expression in fibroblasts of dcSSc patients than in lcSSc patients, however, the differences between the types of SSc were not significant.

Our findings suggest that MIF and CD74 may have an important role in the localized inflammatory process (in the skin) in SSc patients but not at the systemic level, since we observed lower expression of *MIF* and *CD74* mRNA in SSc patients than in CS. The expression of MIF and CD74 was observed in the skin of SSc patients, mainly in keratinocytes and fibroblasts, which are important cells in the tissue remodeling process in SSc. In addition, the overexpression of CD74 observed in biopsies of patients with dcSSc suggests that CD74 could be involved in exacerbated fibrosis in dcSSc, however, their participation is unclear. Additional studies are required in the

different types of SSc to describe the involvement of MIF/CD74 in the fibrotic process of this disease.

Acknowledgments

The study was supported by funding from the National Council of Science and Technology (CONACYT) Grant A1-S-8774 (CONACYT Ciencia Básica-Universidad de Guadalajara) assigned to JFMV. Also, CJH-B was a Ph.D. fellow at CONACYT-Mexico (#301502).

Conflict of interest

The authors declare no conflicts of interest related to this study.

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TABLE AND FIGURES

Table 1 Demographic and clinical characteristics of SSc patients

Variables	SSc (n=20)	CS (n=20)
Demographics		
Age (years)[a]	44.4 ± 17.7	39.6 ± 14.6
Gender[b]		
Female	80 (16)	75 (15)
Male	20 (4)	25 (5)
BMI (kg/m^2)[a]	24.4 ± 3.9	26.9 ± 4.0
Clinical assessment		
Disease subtype[b]		
lcSSc	70 (14)	-
dcSSc	30 (6)	-
Disease evolution, years[c]	4 (0.5-20)	-
Clinical manifestations[b]		
Skin thickening	95 (19)	-
Joint contractures	95 (19)	-
Puffy fingers	75 (15)	-
Raynaud´s phenomenon	70 (14)	-
Telangiectasia	50 (10)	-
Clinical evaluation		
Spanish HAQ-DI, 0-3 scale[a]	0.46 ± 0.44	-
mRSS51[c]	6 (0-30)	-
Drug treatment[b]		
None	55 (11)	100 (20)
Prednisone	35 (7)	-
Methotrexate	35 (7)	-
Chloroquine	15 (3)	-

[a]Data provided in mean ± standard deviation. [b]Data provided in percentage and n. [c]Data provided in median (p5–p95). SSc, systemic sclerosis; BMI, body mass index; dcSSc, diffuse cutaneous systemic sclerosis; lcSSc, limited cutaneous systemic sclerosis; mRSS, modified Rodnan skin score; Spanish HAQ-DI, The Spanish version of health assessment questionnaire disability index.

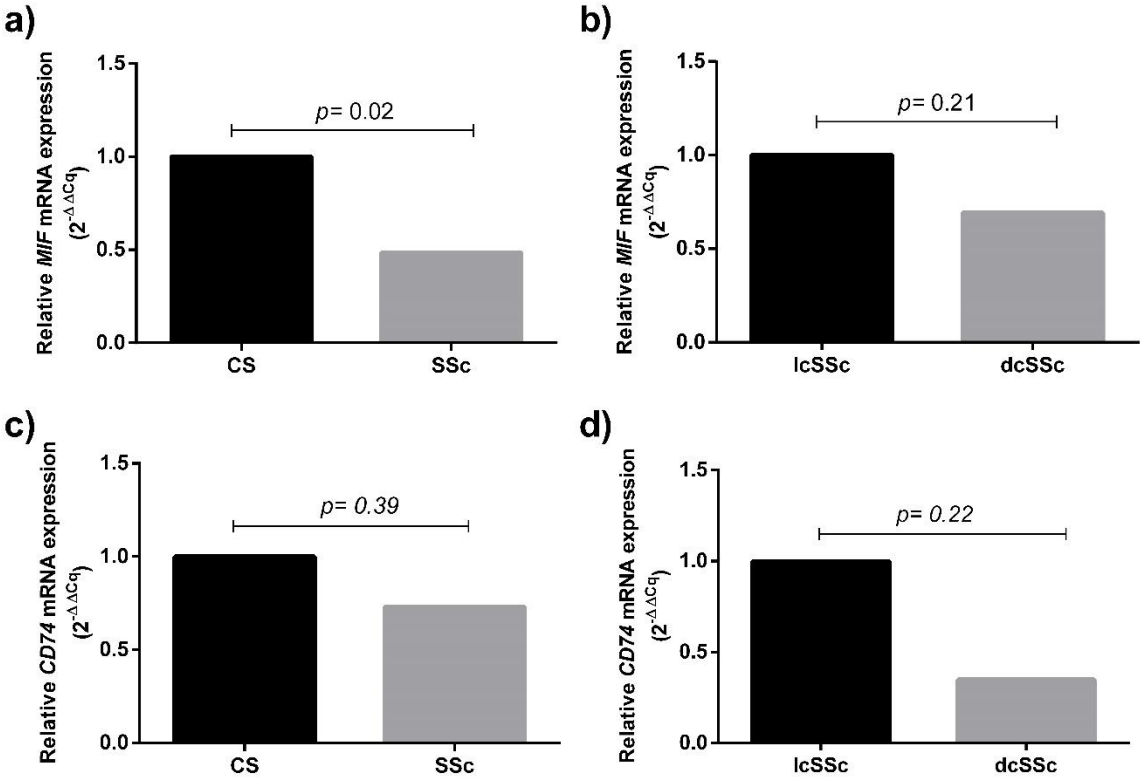

Figure 1. Relative *MIF* and *CD74* mRNA expression. *MIF* and *CD74* mRNA expression in CS and SSc (a-c). *MIF* and *CD74* mRNA expression in SSc patients limited cutaneous (lcSSc) and diffuse cutaneous SSc (dcSSc) (b-d). Relative expression analysis was performed using $2^{-\Delta Cq}$ and $2^{-\Delta\Delta Cq}$ methods and *GAPDH* was the reference gene. Statistical comparisons between groups were made using the Mann-Whitney U test.

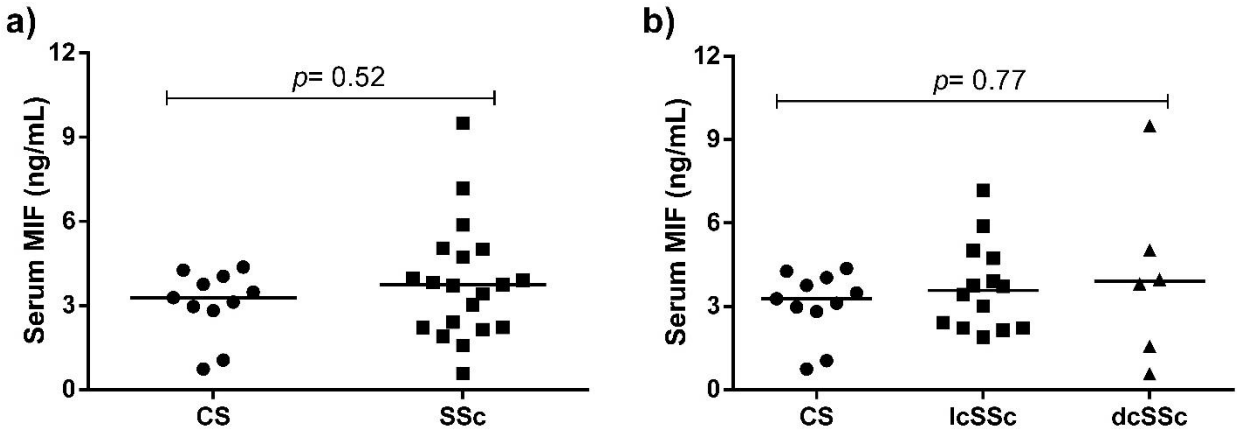

Figure 2. MIF serum levels in CS and SSc patients. a) Comparison of soluble levels of MIF between CS and SSc patients; b) Comparison of soluble levels of MIF between CS, lcSSc, and dcSSc. The values are shown as scatter diagrams. The p-value was calculated with the Kruskal-Wallis test or Mann-Whitney U test, as appropriate. Limited cutaneous systemic sclerosis (lcSSc), diffuse cutaneous SSc (dcSSc), CS (control subjects).

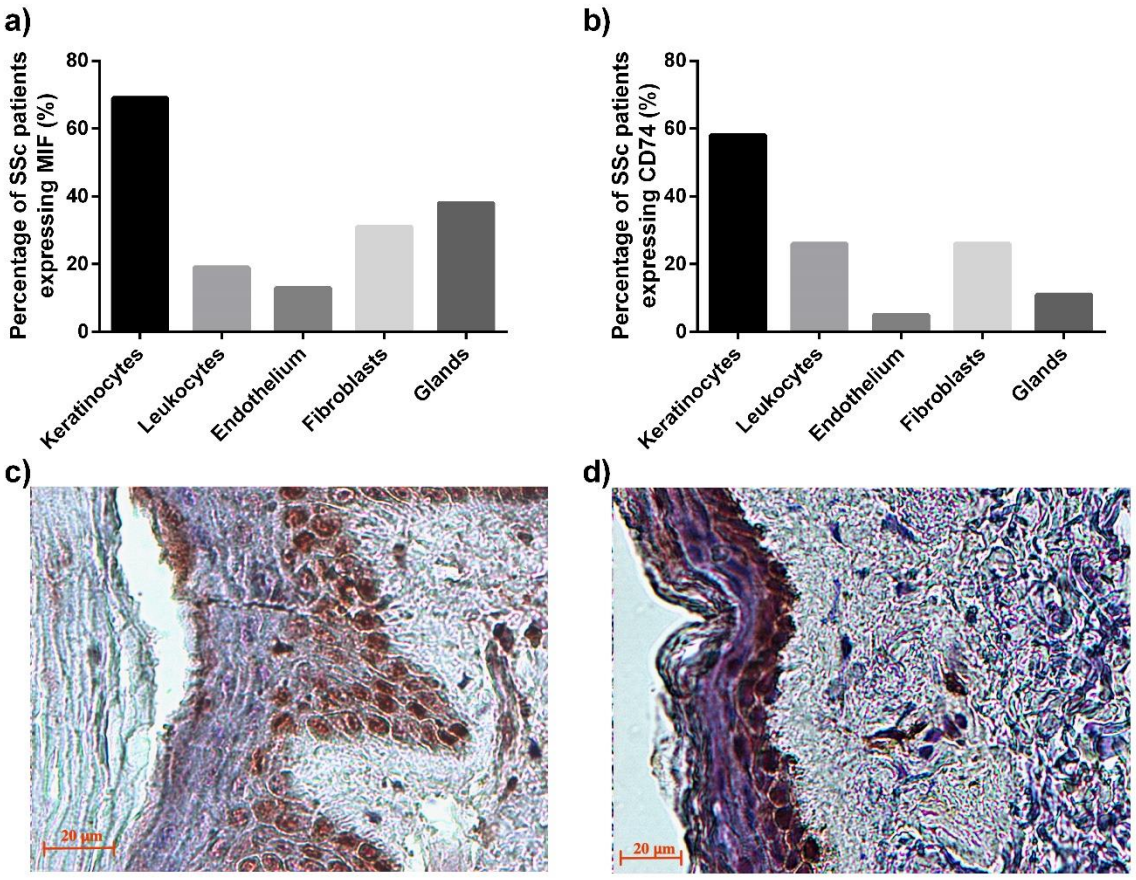

Figure 3. MIF and CD74 expression in skin biopsies. a) Percentage of SSc patients with MIF staining in skin cells; b) Percentage of SSc patients with CD74 staining in skin cells. Images were qualitatively analyzed as positive and negative for each cell type by two pathologists' specialists. Expression of MIF (c) and CD74 (d) in the skin of patients with SSc. Representative sections stained by immunohistochemistry (3,3'-Diaminobenzidine, brown). MIF was expressed in the cytoplasm and nucleus of the keratinocytes in the stratum basale and partially in the stratum spinosum. CD74 was expressed predominately in keratinocytes of the stratum basale.

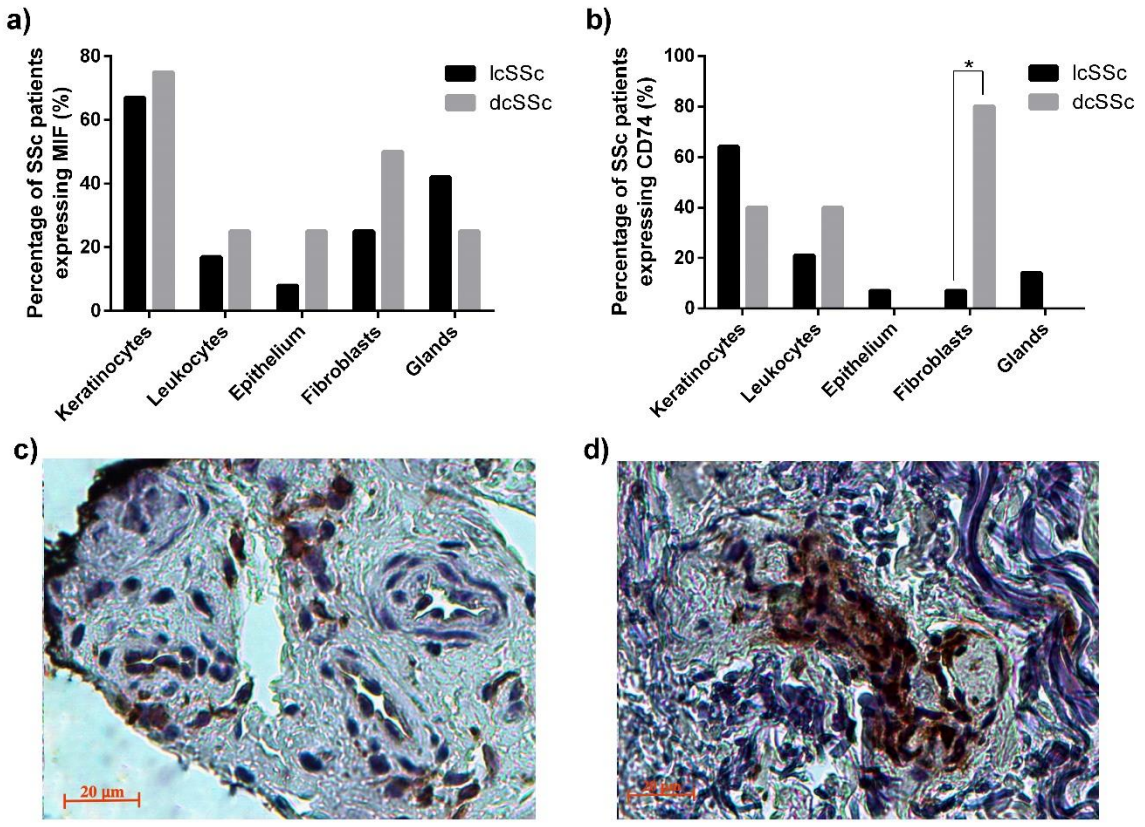

Figure 4. MIF and CD74 expression in skin biopsies by type of SSc. a) Comparison of the percentage of lcSSc or dcSSc patients with MIF positive staining; b) Comparison of the percentage of lcSSc or dcSSc patients with CD74 positive staining. Expression of CD74 in fibroblast of patients with lcSSc (c) and dcSSc (d). Representative sections stained by immunohistochemistry (3,3'-Diaminobenzidine, brown. c) There is a moderate expression of CD74 in scattered fibroblasts and endothelial cells of the reticular dermis in the tissue of a lcSSc patient. d) A group of fibroblasts adjacent to a capillary vessel in the reticular dermis shows strong expression of CD74 in the cytoplasm and nucleus of a dcSSc patient. *Significant differences ($p>0.05$) were observed between lcSSc and dcSSc when comparing the positivity of CD74 staining in fibroblasts. Images were qualitatively analyzed as positive and negative for each cell type by two pathologists' specialists.

DISCUSIÓN

La ES se define como una enfermedad autoinmune, inflamatoria, crónica y multifactorial de etiología desconocida (Barsotti, 2016), sin embargo, puede estar relacionada con factores ambientales, hormonales y genéticos (Ramos, 2015). En los factores ambientales, destacan la exposición a agentes químicos: la sílice y asbestos, la exposición a solventes orgánicos, derivados del benceno e incluso se ha sugerido al tabaquismo (Marie, 2011; Stern&Denton, 2015). En el caso de la población de estudio, no se encontraban expuestos a los factores antes mencionados, sin embargo un porcentaje importante estaba expuesta al humo de leña, que no se ha considerado un factor de riesgo hasta la fecha. No obstante, se ha descrito que fumar aumenta el riesgo de manifestaciones cutáneas, vasculares, gastrointestinales y respiratorios en la ES (Zhang, 2017). Por lo cual sugerimos que estos dos factores pueden tener elementos en común, que deben ser analizados posteriormente. La exposición a pesticidas y fertilizantes presentó un papel importante en esta población de estudio, debido a que no se reconocen como factores de riesgo, pero se ha descrito que existe una marcada correlación entre la ES y la exposición a los metales pesados: antimonio, cadmio, plomo, mercurio, molibdeno, paladio y zinc (Marie, 2017), que son los principales componentes de los pesticidas y fertilizantes utilizados en la agricultura. Por lo anterior, también resulta interesante investigar a fondo el papel de los pesticidas y fertilizantes, como posibles factores de riesgo ocupacional para ES.

Los factores hormonales se han asociado con ES por el incremento en la prevalencia de la enfermedad en mujeres respecto a los hombres (4:1 a 14:1) (Oliver&Silman, 2009). En nuestra población la proporcion de la enfermedad de acuerdo a mujeres y hombres fue de 6:1, lo cual coincide con lo reportado en la bibliografía. Por otro lado, también se ha descrito que hormonas endocrinas como el estradiol podrían participar en el inicio o la progresión de la enfermedad, por sus efectos profibróticos en algunos mediadores células importantes, como IL-1 y TNF-α (Stern&Denton, 2015). Por lo cual se plantea la importancia de evaluar el efecto de las hormona endocrinas en esta poblacion. Por último, otro factor que podría contribuir al aumento de la ES en las mujeres con respecto a los hombres, es la persistencia de células fetales en tejidos maternos (microquimerismo

fetal) pues se ha propuesto como un mecanismo desencadenante para la ES y otras enfermedades autoinmunes. Sin embargo, es necesario realizar estudios más complejos para describir un mecanismo exacto (Oliver&Silman, 2009).

Dentro de los factores genéticos, se han descrito varios polimorfismos asociados con la susceptibilidad y la gravedad de la enfermedad. Los genes que se asocian de manera más consistente se encuentran en el locus *HLA-DR*. Otros genes fuera de la región HLA asociados comprenden *IRF5 (rs607218), STAT4 (rs600558), IL12RB2 (rs601642), PXK (rs611450) y MIF (rs755622)* (Wu, 2006; Bossini-Castillo, 2011; Mayes, 2014; Bossini-Castillo, 2015).

MIF es una citocina inmunorreguladora que inhibe la apoptosis inducida por la activación de p53, induce la secreción de citocinas proinflamatorias tales como TNFα, IL-1β, IL-2, IL-6, IL-8 e IL-12; y regula la proliferación de diferentes tipos de células a través de su unión a sus receptores (Bucala, 2012). El complejo MIF-CD74-CD44 desencadena la activación de diversas vías de señalización, incluidas ERK1/2, AMPK y Akt (Jankauskas, 2019). En la ES, se ha sugerido que MIF podría contribuir a la fibrosis de la piel al inhibir la apoptosis de los fibroblastos dérmicos (Kim, 2008), y aumentar la proliferación de fibroblastos y la síntesis de colágeno (Ningyan, 2015). Sin embargo, la participación de MIF y sus receptores en la patogénesis de la ES ha sido poco estudiada. Para obtener más información sobre el papel de MIF en ES, se evaluó la asociación de los polimorfismos -794 $CATT_{5\text{-}8}$ y -173 G>C con la expresión de MIF (mRNA y proteína) y su correlación con el perfil de citocinas Th1/Th2/Th17 en pacientes con ES del sur de México. Asimismo, se evaluó la expresión sistémica de MIF y CD74 en ES y SC, así como la expresión tisular de estas proteínas en piel de pacientes con ES.

Previamente, se ha descrito en dos poblaciones independientes la asociación del polimorfismo -173 G>C del promotor de *MIF* con ES, de manera más consistente con ESD (Wu, 2006; Bossini-Castillo, 2011). En este estudio, se identificó una asociación significativa de los alelos -794 $CATT_7$ y -173*C de *MIF* y su haplotipo funcional C7 con ES, por lo que sugerimos que los polimorfismos del promotor de *MIF* pueden conferir mayor susceptibilidad para desarrollar ES. En los estudios realizados en poblaciones de América del Norte y Europa (ocho poblaciones europeas) no sólo se confirmó una

asociación entre el alelo C del polimorfismo -173 G>C del promotor de *MIF* con la ES, sino que se observó una mayor frecuencia del alelo de riesgo (C) en el subgrupo de pacientes con ESD (Wu, 2006; Bossini-Castillo, 2011). Una de las limitaciones del presente estudio fue que no se realizó la estratificación por tipo de ES (ESL y ESD), debido a que la ESD tuvo una frecuencia muy baja (14%), lo cual, no permitió un análisis estadístico apropiado.

Con respecto al polimorfismo -794 $CATT_{5-8}$ de *MIF* en ES, sólo ha sido evaluado por Wu et al., en la población norteamericana (Estados Unidos de América y Canadá), pero a diferencia de nuestra población, en este estudio no se observó una asociación entre el polimorfismo y la ES (Wu, 2006). Esta discrepancia podría explicarse por la diferente estructura genética que existe entre ambas poblaciones, ya que la población evaluada por Wu et al. fue una población caucásica de los Estados Unidos de América y Canadá, donde su ascendencia podría ser principalmente europea (Roy-Gagnon, 2011; Bryc, 2015). Respecto a la ascendencia de nuestra población de estudio es predominantemente amerindia (48%), seguida de europea (38%), asiática (10%) y africana (4%) (Martínez-Cortes, 2012). Por lo tanto, es necesario investigar más a fondo el impacto de las variaciones genéticas en el gen *MIF* en poblaciones adicionales, con mayores tamaños de muestra y con una mejor definición de los fenotipos clínicos.

También se investigó la asociación de los polimorfismos de *MIF* con los índices de evaluación clínica, manifestaciones clínicas y autoanticuerpos, pero no se observaron diferencias significativas. No obstante, en población europea se observó una asociación significativa del alelo -173*C del gen *MIF* con la susceptibilidad para desarrollar hipertensión arterial pulmonar (HAP) en pacientes con ESD (Bossini-Castillo, 2017). Del mismo modo, se ha investigado la asociación del polimorfismo -173 G>C de *MIF* con los anticuerpos topoisomerasa I (ATA) y los anticuerpos anti-centromero (ACA), pero no se han observado diferencias significativas (Wu, 2006; Bossini-Castillo, 2011). En otras enfermedades autoinmunes como la AR, se observó que los portadores de los alelos -794 $CATT_7$ y -173*C tenían una alta actividad de la enfermedad, clasificada por el DAS28 (Llamas-Covarrubias et al, 2013).

Posteriormente, para evaluar el efecto de los polimorfismos en la expresión de mRNA de *MIF*, se estratificó a los pacientes de acuerdo al haplotipo homocigoto de susceptibilidad (7C/7C) y al haplotipo de no susceptibilidad (6G/6G). Se observó que los pacientes con el haplotipo de susceptibilidad tenían una mayor expresión de mRNA de *MIF* que los pacientes con el haplotipo de no susceptibilidad. En consecuencia, es importante destacar que está es la primera vez que se evalúa el efecto de los haplotipos de *MIF* a nivel de mRNA en pacientes con ES. Anteriormente, Wu y cols., describieron el efecto de los haplotipos de *MIF,* pero a nivel de proteína. Donde los autores observaron que los pacientes portadores del haplotipo 7C tenían niveles más altos de MIF soluble en comparación con los portadores de haplotipos diferentes a 7C, sin embargo, estas diferencias no fueron estadísticamente significativas (Wu, 2006).

En diversos estudios se ha demostrado que los niveles de MIF están incrementados tanto en suero como sobrenadante de cultivo de fibroblastos dérmicos en pacientes con ES (Becker, 2008; Kim, 2008; Vicent, 2018), incluso al estratificar por tipo de ES, se ha observado que los pacientes con ESD presentan niveles más altos de MIF que los pacientes con ESL y SC (Selvi, 2003; Becker, 2008). Sin embargo, en nuestra población no se observaron estas diferencias significativas al comparar los niveles séricos de MIF entre pacientes con ES y SC, ni al estratificarlos por tipo de ES. Los resultados obtenidos en el presente estudio coinciden con lo reportado por Wu y cols., quienes no observaron diferencias significativas al comparar los niveles séricos de MIF por los tipos de ES, sin embargo, ellos argumentan una tendencia a niveles más altos de MIF en el grupo de pacientes con ESD (Wu, 2006). La discrepancia entre los resultados de los estudios anteriores refleja la variabilidad en los niveles séricos de MIF, que puede verse influida por el tratamiento para la ES (metotrexato, glucocorticoides y cloroquina) (Hong, 2004; Petrovsky, 2003; Aeberli, 2006).

Existen otros factores que pueden modificar los niveles de MIF, como la obesidad o el sobrepeso, a pesar de que ninguno de nuestros grupos de estudio presenta obesidad, si se observó que los SC tienen mayor IMC (incide de masa corporal) que los pacientes con ES (26.5 vs. 24 kg/m^2, respectivamente; p=0.04) (Church, 2005). Dandona y cols., reportaron una correlación positiva entre los niveles séricos de MIF y el IMC (Dandona,

2004). Posteriormente, se confirmó el aumento de los niveles de MIF en plasma, así como el aumento de la expresión del mRNA de *MIF* en individuos obesos en comparación con individuos delgados (Kleemann, 2010). Además, se observó una disminución de los niveles séricos de MIF (hasta un 67%) en sujetos obesos después de una pérdida de peso drástica (Sheu, 2008). Por lo anterior, se sugiere que la pérdida de peso puede ser un factor importante para la disminución de los niveles séricos de MIF en los pacientes, debido a que estos suelen tener signos relacionados con pérdida de peso como la mala absorción, deficiencia de vitaminas y deficiencias nutricionales, producto de las manifestaciones de la enfermedad (dismotilidad esofágica, gastroparesia, microstomia, entre otros) (McFarlane, 2018).

Por otra parte, Corallo y cols., sugieren que el aumento de MIF puede inhibir la apoptosis de los firboblastos dérmicos, y de esta manera producir una fibrosis excesiva en esta enfermedad (Corallo, 2015). También se ha sugerido que MIF podría inhibir la apoptosis en los fibroblastos de forma paracrina, específicamente MIF producido por las células T (Kim, 2008). Por lo anterior, en este estudio se evaluó por primera vez la expresión de mRNA de *MIF* en leucocitos de sangre periférica, contrario a lo que se esperaba, se observó una mayor expresión de mRNA de *MIF* en SC que en pacientes con ES.

En estudios previos, se ha descrito que algunos fármacos antirreumáticos, como la cloroquina, pueden regular negativamente la expresión del mRNA de citocinas proinflamatorias (TNF-α e IL-1β) que se correlacionan positivamente con la expresión de MIF (Karres, 1998). Sin embargo, en este estudio, los resultados no se pueden atribuir al tratamiento, ya que tanto la expresión del mRNA de *MIF* como los niveles séricos de esta citocina se compararon en pacientes con tratamiento y sin tratamiento, pero no se observaron diferencias significativas. No obstante, es importante considerar el hecho de que MIF no sólo se expresa en células de la respuesta inmune, sino que también se expresa en células de la piel y está asociado con la reparación de heridas (Shimizu, 2005).

Aunado a lo anterior, CD74 se ha descrito como un receptor de alta afinidad para MIF (Lindner, 2017), por lo se consideró importante evaluar la expresión del mRNA de *CD74* en leucocitos de sangre periférica en pacientes con ES y SC. Este estudio también es el

primero en evaluar la expresión del mRNA de *CD74* en pacientes con ES. En este sentido, se observó una tendencia a mayor expresión de mRNA de *CD74* en CS que en pacientes con ES, pero no hubo diferencias significativas como en el caso de la expresión de mRNA de *MIF*.

Respecto a las citocinas involucradas en la patogénesis de la ES, se ha observado la participación de las citocinas de los perfiles Th2 y Th17, entre las cuales destacan TGF-β, IL-6, IL-1β, IL-21, IL10 e IL-13 (Meloni, 2009, Yoshizaki, 2010, Rodríguez-Reyna, 2012, O'Reilly, 2012). Se han descrito niveles más altos de las citocinas IL-10, TNF-α y el perfil de citocinas Th17 en suero y condensado de aliento exhalado (CAE) de pacientes con ESD y ESL que en SC (Rolla, 2016). De manera similar, en este estudio se observaron niveles elevados de las citocinas IL-1β e IL-6 que forman parte del perfil de citocinas Th17 en pacientes con ES. Pero no se observaron diferencias significativas en los niveles séricos de estas citocinas de acuerdo al tipo de ES. El aumento de los niveles séricos del perfil de citocinas Th17 se puede explicar por la sobreexpresión de algunas moléculas de adhesión como L-selectina e ICAM, pues ambas regulan la migración de las células Th2 y Th17 a la piel y el pulmón, lo cual conduce al desarrollo de la fibrosis en estos órganos en ES. Por otro lado, P-selectina, E-selectina y PSGL-1 regularán la infiltración de las células Th1, lo que resulta en la inhibición de la fibrosis, sin embargo, esto no ocurre en la ES, ya que estás moléculas se encuentran disminuidas (Yoshizaki, 2010).

Sin embargo, en el perfil de citocinas Th2 no se encontraron diferencias significativas entre los pacientes con ES y SC, donde se midieron IL-10 e IL-4, mientras que en varios estudios se ha descrito consistentemente la participación de las células Th2 en la patogénesis de la ES y los niveles elevados de sus citocinas, como la IL-4 y la IL-13. Se ha descrito que la IL-4 es crítica para la polarización de las células Th2, además de IL-13 que también es necesaria para modular la respuesta Th2 en ES (O'Really, 2012). Por lo anterior, se investigó si existen diferencias en el perfil de citocinas de acuerdo al tratamiento, y se observó que los niveles de IL-4 están disminuidos en el grupo de pacientes con tratamiento en comparación con aquellos pacientes que no tomaban tratamiento. Se sugiere que el tratamiento podría estar afectando los niveles de IL-4, según un estudio reciente que establece que los medicamentos como los

glucocorticoides (dexametasona) producen sinergia con fármacos biológicos dirigidos a IL-4 (F8-IL4) en el tratamiento de afecciones inflamatorias crónicas (Schmid, 2018).

Así mismo, se ha observado que MIF se asocia con la inmunomodulación de los perfiles de citocinas Th1, Th2 y Th17 en enfermedades inflamatorias. En nuestro estudio observamos una correlación positiva entre los niveles solubles de MIF y las citocinas de los perfiles Th1 (INFγ y TNFβ) y Th17 (IL-17A, IL-17F, IL-1β e IL-21) en pacientes con ES, pero no en SC. Sin embargo, hasta la fecha sólo se ha descrito una correlación positiva del MIF con el TGF-β en la ES (Kim, 2008). En cambio, nuestro grupo de investigación ha evaluado esta correlación en otras enfermedades autoinmunes, como AP y LEG. En AP, se evaluó la correlación entre la expresión de mRNA de *MIF* y *TNF-*α con los perfiles de citocinas Th1, Th2 y Th17, donde se observó que la alta expresión del mRNA de *TNF-*α correlaciona positivamente con el aumento de las citocinas de los perfiles Th1 (INF-γ y TNF-α) y Th17 (IL-17 e IL-22), pero esta correlación no fue similar en la expresión *MIF* (Bautista-Herrera, 2017). Mientas que en células mononucleares de sangre periférica (PBMCs) de pacientes con LEG y SC se observó que MIF induce la respuesta inflamatoria en condiciones fisiológicas (SC) y patológicas (LES) con un predominio del perfil Th17 en SC y un aumento en TNF-α e IL-6 en LEG activo (De la Cruz-Mosso, 2018). Esto sugiere que MIF es una citocina inmunomoduladora que podría no tener predominio hacia un perfil Th específico.

Previamente, se ha descrito el papel del MIF es crucial en la aceleración de la cicatrización de heridas, pues responde a la lesión tisular regulando las fases inmunológica e inflamatoria del proceso de curación (Shimizu, 2005; Zhao, 2005). También se ha descrito que los cultivos de fibroblastos de heridas de la piel, producen una mayor cantidad de MIF en respuesta a estímulos y, curiosamente, este factor es importante en la migración no sólo de los fibroblastos, sino también tiene un efecto quimiotáctico sobres los queratinocitos y la regeneración de la piel después de la herida (Abe, 2000; Pazyar, 2013). En conjunto, estos resultados podrían sugerir que el MIF contribuye al proceso de cicatrización de la herida del tejido de la piel, incluida la remodelación tisular y la fibrosis que son características del SSc.

Para obtener más información sobre el papel de MIF y CD74 en piel de pacientes con ES, examinamos su expresión en biopsias de piel realizadas por inmunohistoquímica, donde se encontró que MIF y CD74 se expresaron en un mayor porcentaje en queratinocitos seguidos de fibroblastos, glándulas sudoríparas, células mononucleares infiltrantes y células endoteliales en la piel de pacientes con ES. Estos hallazgos coinciden con lo informado por Selvi, et al., donde observaron la tinción con MIF en queratinocitos, fibroblastos, glándulas sudoríparas, células mononucleares infiltrantes y células endoteliales. Sin embargo, estos autores no evaluaron la presencia del receptor CD74 (Selvi, 2003).

Por otro lado, Corallo y cols., fueron los primeros en evaluar el complejo receptor de MIF (CD74/CD44) en biopsias de piel de pacientes con ES. En este estudio, se informó una expresión elevada de MIF en queratinocitos, fibroblastos, endotelio, glándulas sebáceas/sudoríparas de pacientes con ESL/ESD. Asimismo, detectaron una inmunotinción débil de MIF en la piel de controles y en la piel no afectada de pacientes con ESL. Con respecto al complejo del receptor de MIF, CD74/CD44, no encontraron diferencias entre los controles y los pacientes con ESL/ESD en piel afectada y no afectada (Corallo, 2015).

Por último, se observó un mayor porcentaje de expresión de CD74 en fibroblastos de pacientes con ESD que en pacientes con ESL. En contraste, Corallo, y cols., no encontraron diferencias en el análisis de los receptores CD74/CD44 entre los controles y los pacientes con ESL/ESD, afectados y no afectados de la piel (Corallo, 2015). Con respecto a la expresión de MIF en fibroblastos dérmicos de pacientes con ESL y ESD, parece tener el mismo comportamiento que CD74, sin embargo, las diferencias en la expresión de MIF no fueron significativas.

Finalmente, se propone un modelo de integración de los resultados de este trabajo de investigación en pacientes con ES (Figura 1). Donde se encontró una asociación significativa de los alelos -794 $CATT_7$ y -173*C de *MIF* y su haplotipo funcional C7 con mayor susceptibilidad a desarrollar ES en pacientes del sur de México (Guerrero), así como, una asociación del haplotipo C7 con una alta expresión del mRNA. Además, los resultados obtenidos mostraron niveles elevados de las citocinas IL-6 e IL-1β en ES, así

como una correlación positiva entre los niveles de MIF y los perfiles de citocinas Th1 y Th7 en los pacientes con ES, pero no en los SC. También se observó la expresión de MIF y su receptor CD74 a nivel tisular, principalmente en queratinocitos y fibroblastos dérmicos. El receptor CD74 se expresa principalmente en los fibroblastos dérmicos de pacientes con ES, mientras que MIF sólo mostró una tendencia a mayor expresión en estos pacientes. En resumen, podemos observar que tanto MIF como su receptor CD74, podrían participar en la patogénesis de la ES.

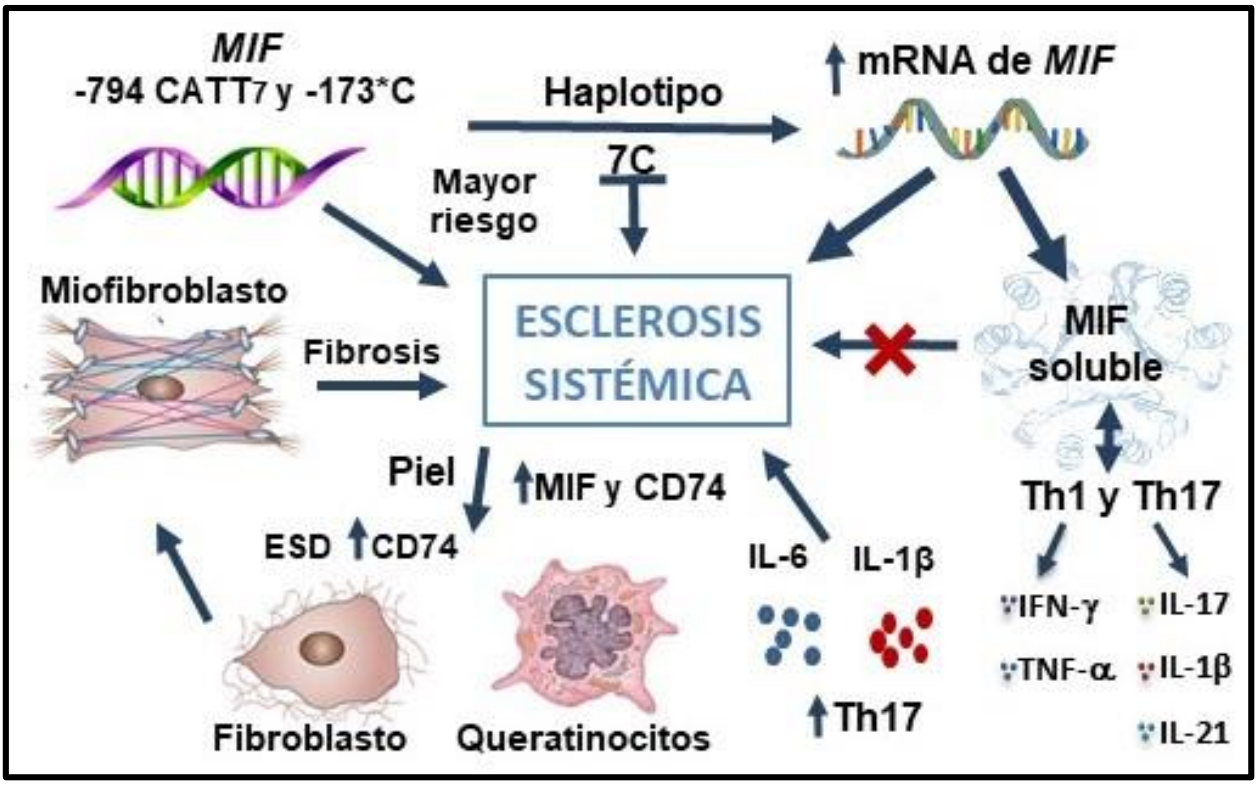

Figura 1. Modelo de integración de resultados. Los polimorfismos de *MIF* -794 $CATT_7$ y -173*C y su haplotipo funcional 7C se asociaron con susceptibilidad para desarrollar ES, y una mayor expresión de mRNA de *MIF* en pacientes con ES. MIF soluble correlacionó positivamente con citocinas de los perfiles Th1 y Th17 en pacientes con ES. Las citocinas del perfil Th17 (IL-6 e IL-1β) se encontraron incrementadas en los pacientes con ES. En piel, se observó una expresión elevada de MIF y CD74, principalmente en queratinocitos y fibroblastos, mientras que CD74 se sobreexpresó en fibroblastos dérmicos, las células responsables de la producción de matriz extracelular (fibrosis), de pacientes con ESD.

CONCLUSIONES

Este estudio proporciona evidencia de que los polimorfismos individuales y el haplotipo funcional (C7) de *MIF* están asociados con la susceptibilidad para desarrollar ES y la alta expresión del mRNA de *MIF* en la población del sur de México.

R, los resultados obtenidos sugieren que las citocinas IL-1β e IL-6 que forman parte del perfil de citocinas Th17 se encuentran incrementadas en pacientes con ES, lo cual coincide con el papel de las células Th17 en la patogénesis de la ES. También se sugiere MIF podría inducir una respuesta inflamatoria con predominio del perfil de citocinas Th17 modulando el aumento de las citocinas IL-17A, IL-17F, IL-1β e IL-21.

Los hallazgos del presente estudio sugieren que MIF y CD74 pueden tener un papel importante en el proceso inflamatorio localizado (la piel), así como en el proceso fibrótico en pacientes con ES. Sin embargo, a nivel sistémico MIF y CD74 no parecen tener el mismo comportamiento, ya que no se observó una mayor expresión de *MIF* y *CD74* en pacientes con ES.

En piel de los pacientes con ES, la expresión de MIF y CD74 se expresa principalmente en queratinocitos y fibroblastos, las cuales son células importantes en el proceso de remodelación de tejidos en ES. Además, el receptor CD74, se sobreexpresa en las biopsias de pacientes con ESD, lo que sugiere que CD74 podría estar involucrado en la fibrosis exacerbada en ES, sin embargo, su participación aún no es clara. Por su parte, MIF tuvo un comportamiento similar pero las diferencias no fueron significativas entre los dos tipos de ES. Por lo anterior, se concluye que se requieren estudios adicionales en los diferentes tipos de ES para describir la participación de MIF/CD74 en el proceso fibrótico de esta enfermedad.

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ANEXOS

Anexo 1. Carta de consentimiento infomado

Fecha:_____ **No. de expediente:**_____ **Folio:**_____

CARTA DE CONSENTIMIENTO INFORMADO

Por medio de la presente acepto participar en el proyecto de investigación para la identificación de variantes genéticas de *MIF* y presencia o ausencia de *KIR* y *HLA* en pacientes con esclerodermia sistémica del estado de Guerrero.

Se me ha explicado que mi participación es voluntaria y consiste en **responder un cuestionario y una toma de sangre periférica por punción venosa**. Declaro que se me ha informado que no representa mayor riesgo que **la posible formación de un hematoma en la zona de punción.**
El investigador se comprometió a responder mis preguntas y aclarar cualquier duda respecto a los procedimientos que se realizarán o cualquier otro asunto relacionado con la investigación, además me aseguró que mi identidad y mis datos serán manejados de forma confidencial y no serán divulgados en presentaciones o publicaciones que deriven de este estudio.

De igual forma acepto que las muestras se guarden en congelación y se utilicen en otros estudios posteriores, respetando la confidencialidad de los datos y solo si esas investigaciones son aprobadas por los comités de investigación y de ética de la Universidad de Guadalajara y/o de la Universidad Autónoma de Guerrero.

NOTA: para cualquier emergencia o duda se podrá comunicar con la persona responsable Christian Johana Baños Hernández al número telefónico: (044) 7471493487.

Paciente

_____
Nombre y firma

Responsables

_____ _____

M. en C. Christian Johana Baños Hernández

C. P. 8251355

Lic. en Bioanálisis Andrea Carolina Machado Sulbaran

Investigadores Principales

_____ _____

Dr. en C. José Francisco Muñoz Valle
Doctor en Biología Molecular en Medicina

Dr. José Eduardo Navarro Zarza
Medicina Interna - Reumatología

Anexo 2. Encuesta

Fecha:_____ **No. de expediente:**_____ **Folio:**_____

DATOS DEL PACIENTE

Nombre: _____ Género: _____

Edad: _____ Edo. Civil:_____ Ocupación:_____

Dirección (Calle y colonia): _____

Ciudad y Estado: _____ Tel.:_____

Atención del paciente: Seguro Popular: _____ Consulta Privada: _____ Otro: _____

Sus padres son de Guerrero: Si_____ No_____ Sus abuelos son de Guerrero: Si_____ No_____

Tipo de Esclerodermia: ESL:_____ ESD:_____

Edad de inicio _____ Fecha de diagnóstico:_____ Tiempo de evolución: _____

ANTECEDENTES

Antecedentes Familiares:

Artritis reumatoide ☐ D.M. tipo 1 ☐ Esclerodermia ☐ L.E.S. ☐ Cáncer ☐

Otro: _____

Factores de riesgo

Trabaja o trabajo en: Construcción ☐ Minería ☐ Industria ☐ Especifique:_____

Alguien de su casa: Construcción ☐ Minería ☐ Industria ☐ Especifique:_____

Cerca de su casa: Construcción ☐ Minería ☐ Industria ☐ Especifique:_____

Ha vivido anteriormente cercar de alguno de estos sitios/distancia:_____

Alguien de su casa a vivido cerca de alguno de estos sitios/distancia:_____

No. de habitantes en su casa Uno ☐ Dos ☐ Tres ☐ Cuatro o mas ☐

Lugar de origen Rural ☐ Urbano ☐ Nombre del lugar:_____

Lugar de residencia Rural ☐ Urbano ☐ Nombre del lugar:_____

Tabaquismo previo/actual Si ☐ No ☐ Años/No. de cigarrillos diario: _____

Cocina con leña Si ☐ No ☐ Años/Hrs. diario: _____

Utiliza /utilizo drogas Si ☐ No ☐ ¿Cuáles? _____

Antecedentes patológicos

AR ☐ Sjogrën ☐ D.M. tipo 1 ☐ Gastritis ☐

Dislipidemias ☐ Cáncer ☐ Otros: _____

Fecha:_____ **No. de expediente:**_____ **Folio:**_____

SIGNOS Y SÍNTOMAS					
	Si	**No**		**Si**	**No**
Fenómeno de Raynaud			Úlcera dactilar		
Calcinosis			Dedos inflamados		
Disfunción esofágica			Hipertensión arterial pulmonar		
Esclerodactilia			Enfermedad pulmonar intersticial		
Telangiectasias			Artritis		
Otros:			Apertura oral		

LABORATORIO					
VSG		Valores de biometría hemática		Química sanguínea	
pCr		Leucocitos		Glucosa	
FR		Eritrocitos		Colesterol	
anti-nucleares		Hematocrito		TGO	
anti-centromero		Hemoglobina		TGP	
anti-Scl-70		Plaquetas		Albumina	
Otros:					

TRATAMIENTO									
	Si	No	Dosis	Tiempo		Si	No	Dosis	Tiempo
Metotrexate					Calcio/calcitriol				
Cloroquina					Omeprazol				
Sulfazalacina/AZF					AINEs				
MPS					Paracetamol				
Prednisona					Naproxeno				
Ciclofosfamida					Diclofenaco				
Ac. Fólico					Otros				
Otros:									

PA:_____ Peso (Kg): _____ Talla (Cm): _____ IMC: _____

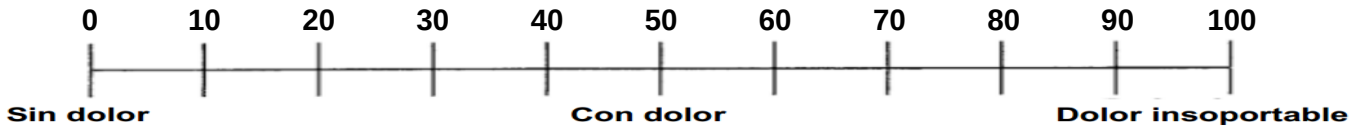
Anexo 3. Health Assessment Questionnaire (HAQ)

Fecha:_____ **No. de expediente:**_____ **Folio:**_____

INSTRUCCIONES: POR FAVOR MARQUE CON UNA "X" LA OPCIÓN QUE MEJOR DESCRIBA SU SITUACIÓN ACTUAL.

ACTIVIDAD	¿PUEDE….	SIN DIFICULTAD	CON DIFICULTAD	CON AYUDA	NO PUEDE
Vestirse y arreglarse	Sacar la ropa del closet (ropero) y cajones?	☐	☐	☐	☐
	Vestirse solo (a) (inclusive, abotonarse y usar cierre)?	☐	☐	☐	☐
	Lavarse el cabello?	☐	☐	☐	☐
Levantarse	Levantarse de una silla recta sin apoyarse con los brazos?	☐	☐	☐	☐
Comer	Cortar la carne?	☐	☐	☐	☐
	Llevarse a la boca un vaso con liquido?	☐	☐	☐	☐
Caminar	Caminar en terreno plano?	☐	☐	☐	☐
Higiene	Lavar y secar todo su cuerpo?	☐	☐	☐	☐
	Abrir y cerrar las llaves del agua?	☐	☐	☐	☐
	Sentarse y levantarse de la taza del baño?	☐	☐	☐	☐
	Peinarse?	☐	☐	☐	☐
Alcance	Alcanzar y bajar una bolsa de 2 Kg que esté por arriba de su cabeza ?	☐	☐	☐	☐
Agarre	Abrir las puertas de un carro?	☐	☐	☐	☐
	Destapar un frasco no muy apretado?	☐	☐	☐	☐
	Escribir? (No puede por causas ajenas a su artritis) ☐	☐	☐	☐	☐
Actividad	Manejar un carro o coser en máquina de pedales? (No puede por causas ajenas a su artritis) ☐	☐	☐	☐	☐
	Salir de compras?	☐	☐	☐	☐

Por favor marque con una "X" lo correspondiente a su situación actual de dolor:

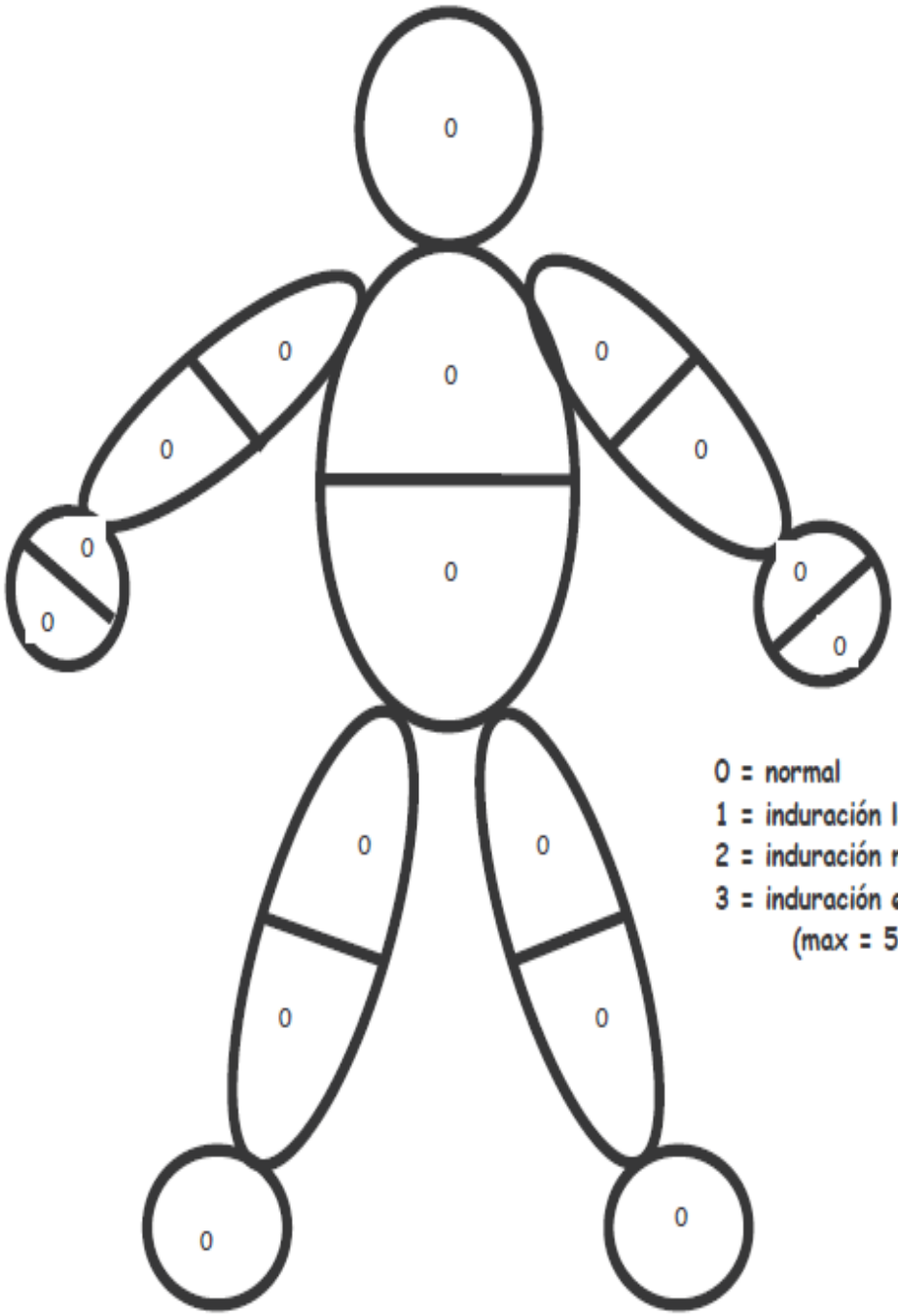


Durante la última semana su estado de salud ha sido:

Anexo 4. Rodnan modificado

Nombre:
Nº Historia:
Fecha:
TSS:

Total Skin Score
(Rodman modificado)
(17 áreas)



0 = normal
1 = induración leve (Pliegue fácil)
2 = induración moderada (Con dif)
3 = induración extrema (Imposibl)
(max = 51)