UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE ESTUDIOS SUPERIORES IZTACALA

BIOMEDICINA

"ESTUDIO DE LA INFLUENCIA DE MIF EN PERIODONTITIS EXPERIMENTAL, PREVIO Y DURANTE LA GESTACIÓN EN UN MODELO MURINO"

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS BIOLÓGICAS

PRESENTA:

M en C. BETSAIDA JULIETA ORTIZ SANCHEZ

TUTORA PRINCIPAL DE TESIS: DRA. MIRIAM RODRÍGUEZ SOSA
FACULTAD DE ESTUDIOS SUPERIORES IZTACALA. UNAM

COMITÉ TUTOR: DRA. LETICIA MORALES LEDESMA
FACULTAD DE ESTUDIOS SUPERIORES ZARAGOZA, UNAM
DR. LUIS ARTURO BAIZA GUTMAN
FACULTAD DE ESTUDIOS SUPERIORES IZTACALA. UNAM

Los Reyes Iztacala, Tlalnepantla, Estado de México, marzo 2024

UNAM – Dirección General de Bibliotecas
Tesis Digitales
Restricciones de uso

DERECHOS RESERVADOS ©
PROHIBIDA SU REPRODUCCIÓN TOTAL O PARCIAL

Todo el material contenido en esta tesis esta protegido por la Ley Federal del Derecho de Autor (LFDA) de los Estados Unidos Mexicanos (México).

El uso de imágenes, fragmentos de videos, y demás material que sea objeto de protección de los derechos de autor, será exclusivamente para fines educativos e informativos y deberá citar la fuente donde la obtuvo mencionando el autor o autores. Cualquier uso distinto como el lucro, reproducción, edición o modificación, será perseguido y sancionado por el respectivo titular de los Derechos de Autor.

UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE ESTUDIOS SUPERIORES IZTACALA

BIOMEDICINA

"ESTUDIO DE LA INFLUENCIA DE MIF EN PERIODONTITIS EXPERIMENTAL, PREVIO Y DURANTE LA GESTACIÓN EN UN MODELO MURINO"

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS BIOLÓGICAS

PRESENTA:

M en C. BETSAIDA JULIETA ORTIZ SANCHEZ

TUTORA PRINCIPAL DE TESIS: DRA. MIRIAM RODRÍGUEZ SOSA
FACULTAD DE ESTUDIOS SUPERIORES IZTACALA. UNAM

COMITÉ TUTOR: DRA. LETICIA MORALES LEDESMA
FACULTAD DE ESTUDIOS SUPERIORES ZARAGOZA, UNAM

DR. LUIS ARTURO BAIZA GUTMAN
FACULTAD DE ESTUDIOS SUPERIORES IZTACALA. UNAM

Los Reyes Iztacala, Tlalnepantla, Estado de México, marzo 2024

COORDINACIÓN GENERAL DE ESTUDIOS DE POSGRADO
COORDINACIÓN DEL POSGRADO EN CIENCIAS BIOLÓGICAS
FACULTAD DE ESTUDIOS SUPERIORES IZTACALA
OFICIO: CGEP/CPCB/FESI/0174/2024
ASUNTO: Oficio de Jurado

M. en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Subcomité de Biología Experimental del Posgrado en Ciencias Biológicas, celebrada el día **27 de noviembre de 2023** se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la estudiante **ORTÍZ SÁNCHEZ BETSAIDA JULIETA** con número de cuenta **93535087** con la tesis titulada **"ESTUDIO DE LA INFLUENCIA DE MIF EN PERIODONTITIS EXPERIMENTAL, PREVIO Y DURANTE LA GESTACIÓN EN UN MODELO MURINO",** realizada bajo la dirección de la DRA. MIRIAM RODRÍGUEZ SOSA**,** quedando integrado de la siguiente manera:

Presidente:	DRA. ANA LILIA GARCÍA HERNÁNDEZ
Vocal:	DRA. GLORIA LUZ PANIAGUA CONTRERAS
Vocal:	DR. JOSÉ LUIS MARAVILLAS MONTERO
Vocal:	DR. JULIO CÉSAR CARRERO SÁNCHEZ
Secretario:	DRA. LETICIA MORALES LEDESMA

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 12 de febrero de 2024

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

c. c. p. Expediente del alumno

AGNS/GGM/EARR/ggm

Agradecimientos Institucionales

Posgrado en Ciencias Biológicas, UNAM.

Durante la realización de la presente tesis de investigación se contó con una parte del apoyo de los proyectos:

Universidad Nacional Autónoma de México, Dirección General de Asuntos del Personal Académico en su Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica **(PAPIIT), proyecto [IN-217021].**

Consejo Nacional de Ciencia y Tecnología **(CONAHCyT), proyecto 152224.**

Consejo Mexiquense de Ciencia y Tecnología **(COMECyT) proyecto [FICDTEM-2021-072]**

Beca **COMECyT 2019**

Apoyo PAEP para asistencia a Congresos y Cursos

Agradezco la asesoría brindada a lo largo del desarrollo de la presente tesis de Doctorado a mi comité tutor:

Dra. Miriam Rodríguez Sosa

Dra. Leticia Morales Ledesma

Dr. Luis Arturo Baiza Gutman

Agradecimientos a título Personal

Jurado asignado por el comité del Posgrado en Ciencias Biológicas para la evaluación del Presente trabajo:

Presidente: Dra. Ana Lilia García Hernández

Vocal (1): Dra. Gloria Luz Paniagua Contreras

Secretario: Dra. Leticia Morales Ledesma

Vocal (2): Dr. José Luis Maravillas Montero

Vocal (3): Dr. Julio César Carrero Sánchez

Mtra. Imelda Juárez Avelar, Dra. Mónica G. Mendoza Rodríguez, Dra. Yolanda I. Chirino, Dra. Gloria Paniagua, Dr. Eric Monroy, Biol. Ulises Andrade, gracias por sus contribuciones para la realización de este trabajo de Doctorado, por sus enseñanzas y apoyo.

Mil gracias a mi familia, mis padres Armando y Esther, mis hermanos Isaac y Armando, Isa y Bere, mis pilares, mi gran apoyo en la vida, los amo infinitamente.

Dra. Miriam, gracias por su apoyo, dedicación, visión y orientación este objetivo alcanzado, parece que fue apenas hace unos días que esta aventura comenzó… Gracias por la oportunidad, por haberme aceptado como su estudiante y ahora como su colaboradora, de corazón gracias.

Ime… muchas gracias por tu paciencia, por tus porras, tus regaños, tu guía, por ser siempre un apoyo, pero sobre todo por tu amistad, eres una persona increíble e importante en mi vida.

Dra. Mony, gracias por tu amistad, por enseñarme y ayudarme con la biología molecular.

Dr. JL, gracias por tu amistad, por confiar y apoyarme, por creer.

Lupita y Vero, gracias por aguantarme, cada una en su trinchera ha tenido que “lidiar” conmigo, su apoyo y amistad ha permitido que alcance esta meta. Sin ustedes habría sido mucho más complicado, estoy eternamente agradecida.

Dra. Rossana, gracias por su apoyo y su confianza han sido de verdad invaluables.

Enrique, Fernanda, Andrés, Karminne, Mariana, Montserrat, mil gracias por su trabajo y dedicación, fueron un gran apoyo durante este proceso.

Thalía, Víctor, Tona, Alicia, Alexia, Danae, Ulises, Vero, Daniel, Enrique, Sonia, y seguro olvido algún nombre, gracias, compañeros y amigos, por el aprendizaje, por las vivencias y por coincidir en la vida de ciencia.

Finalmente, gracias a la vida por la oportunidad de creer y crecer cada día.

Dedicatoria

A mi familia que siempre me ha brindado su amor y apoyo.

Armando, Esther, Armando, Isaac y Sacky los amo infinitamente.

"… Cuando un hombre de ciencia busca conocimientos, aun no hallándolos en su totalidad, descubre fragmentos muy importantes, que son precisamente los que constituyen la ciencia"

Claude Bernard (1865).

Índice

Agradecimientos institucionales	III
Agradecimientos a título personal	IV
Dedicatoria	VI
Índice	VII
Índice de figuras	IX
Abreviaturas	X
Resumen	1
Abstract	3
1. Introducción	4
2. Objetivos	6
2.1 Objetivo General	6
2.2 Objetivos Particulares	6
3. Antecedentes	7
3.1 Microbioma Oral en la enfermedad periodontal y la gestación	8
3.2 Respuesta inmune durante la enfermedad periodontal	8
3.3 Hormonas sexuales y la enfermedad periodontal	10
3.4 Factor inhibidor de la migración de macrófagos (MIF) y la enfermedad periodontal	11
3.5 Metaloproteinasas (MMPs) en la enfermedad periodontal	12
4. Metodología	14
5. Análisis estadístico	16
6. Justificación	17
7. Hipótesis	17
8. Resultados	18
8.1 Los tejidos periodontales de los ratones con periodontitis pregestacional expresaron más MIF en comparación con los ratones con periodontitis	18
8.2 Los ratones Mif-/- desarrollaron menos signos clínicos en periodontitis Pregestacional	19
8.3 La deleción de Mif disminuye el daño de tejidos periodontales y reduce la	19

pérdida de inserción clínica (CAL) en periodontitis pregestacional

8.4 Los ratones *Mif-/-* muestras reorganización de las fibras de colágena 21

8.5 La periodontitis pregestacional inducida en ratones Mif-/- muestran disminución en la expresión de MMP-2 y MMMP-13 durante la gestación 22

8.6 La periodontitis inducida en ratones Mif-/- expresan menores niveles de citocinas inflamatorias 24

8.7 La periodontitis pregestacional en ratones Mif-/- presenta menor actividad de MMP-13 25

8.8 La periodontitis pregestacional ratones Mif-/- presenta mayor actividad de MMP-2 26

8.9 La periodontitis no aumenta la concentración de IFN-γ, TNF-α, IL-6 e IL-10 en suero 27

9. Discusión 30

10. Conclusión 33

11. Referencias Bibliográficas 34

12. Anexo I Articulo Requisito 39

13. Anexo II Articulo Revisión 52

14. Anexo III Técnicas 66

Índice de figuras y cuadros

Fig. 1. Fig. 1. Expresión de RNA de receptores tipo Toll (TLRs) en diferentes células de tejidos periodontales 9

Fig 2. La periodontitis induce la expresión de MIF y el embarazo induce la sobreexpresión del MIF en la enfermedad periodontal previa a la gestación. 18

Fig. 3. Los ratones *Mif-/-* desarrollan síntomas clínicos atenuados en la enfermedad periodontal pregestacional 19

Fig. 4. Los ratones *Mif-/-* muestran menor daño al tejido periodontal y pérdida de inserción que los ratones wild-type en el modelo de periodontitis pregestacional 21

Fig. 5. La deleción del MIF favorece la conservación del colágeno y del epitelio de la bolsa periodontal 22

Fig. 6. Los ratones *Mif-/-* inducidos por periodontitis pregestacional muestran regulación negativa de la expresión de MMP-2 y MMP-13 durante la gestación 24

Fig. 7. La eliminación de MIF disminuye la expresión de *tnf-α* e *ifn-γ* pero no la expresión de *il-17* en la periodontitis 25

Fig. 8. La periodontitis pregestacional presenta menor actividad de MMP-13 en ratones *Mif-/-* 26

Fig. 9 La periodontitis pregestacional presenta mayor activación de MMP-2 en ratones *Mif-/-* 27

Fig. 10. La periodontitis no aumenta la concentración de IFN-γ, TNF-α, IL-6 e IL-10 en suero 29

Abreviaturas

EP	Enfermedad periodontal
PT	Periodontitis
MIF	Factor de inhibición de migración de macrófagos
WT	Silvestre
Mif-/-	Deficiente de MIF
PGPD	Periodontitis pregestacional (Pregestational periodontal disease)
P	Gestantes (Pregnant)
CTL	Control
MMPs	Metaloproteinasas
MMP-2	Metaloproteinasa 2
MMP-13	Metaloproteinasa 13
TNF-α	Factor de necrosis tumoral α
IFN-γ	Interferón γ
Th-17	Linfocitos T colaboradores 17
IL–6	Interleucina 6
IL–10	Interleucina 10
ELISA	Enzyme-Linked ImmunoSorbent Assay (ensayo por inmunoadsorción ligado a enzimas)
IL–17	Interleucina 17
Treg	Linfocitos T reguladores
PAMPs	Patrones moleculares de los patógenos
LPS	Lipopolisacáridos
DAMPs	Patrones moleculares de daño
PRRs	Receptores de reconocimiento de patrones
TLR	Receptores tipo Toll
NFκB	Factor nuclear κB
AP-1	Factor activador de la proteína 1
MAPK	Proteína quinasa activada por mitógenos

Mϕ Monocitos/macrófagos

DCs Células dendríticas

IL-1α Interleucina 1α

IL-1β Interleucina 1β

IL-8 Interleucina 8

MMP-1 Metaloproteinasa 1

MMP-3 Metaloproteinasa 3

IDO Indoleamina 2,3-dioxigenasa

TGF-β Factor de crecimiento transformante β

MMP-3 Metaloproteinasa 3

RANKL Receptor activator of NF-κB ligand

AO's Anticonceptivos orales

GCs Glucocorticoides

CD74 Cadena invariante del complejo principal de histocompatibilidad clase II

H&E hematoxilina y eosina

CAL Clinical Attachment Level

UCE Unión cemento–esmalte

RFI Unidades de fluorescencia relativa

qPCR PCR cuantitativa

Resumen

La enfermedad periodontal (EP) es una alteración inflamatoria de los tejidos periodontales de etología heterogénea que afecta encía, ligamento periodontal, cemento radicular y hueso alveolar, con la consecuente pérdida de inserción clínica de los órganos dentarios. La EP una de las enfermedades más comunes, aproximadamente el 60% de la población a nivel mundial presenta algún grado de esta enfermedad. Esta condición afecta a más de 23% de mujeres en edades entre los 23 y los 54 años, y se ha reportado presente en el 56% de las mujeres gestantes.

Por otro lado, el factor inhibidor de la migración de macrófagos (MIF) es una citocina proinflamatoria que se encuentra significativamente elevada en periodontitis, así como al inicio y al final de la gestación. Aunque la periodontitis suele presentarse con mayor gravedad durante la gestación, no se ha establecido la participación del MIF en el desarrollo y severidad de la periodontitis durante la gestación. Para profundizar sobre la participación de MIF en la exacerbación de la EP durante la gestación, en este estudio se desarrolló un modelo murino de EP. Utilizamos ratones WT y *Mif-/-* con fondo genético BALB/c. Para inducir la periodontitis (PT) se colocó una sutura nylon 6-0 alrededor del segundo molar superior derecho. Para generar la periodontitis previa a la gestación (PGPD) la PT fue inducida dos semanas previas a la cruza. Evaluamos los cambios histológicos, realizamos un análisis histométrico de la pérdida de inserción clínica, la expresión y activación de MMP-2 y MMP-13 por inmunofluorescencia y zimograma, así como concentración del factor de necrosis tumoral (TNF)-α, interferón (IFN)-γ, Interleucina (IL)-6 e IL-10 en suero por ELISA y la expresión relativa *in situ* de *mif, tnf-α, ifn-γ e il-17*por qPCR.

Nuestros resultados muestran que los tejidos periodontales de los ratones PGPD WT producen 2 veces más MIF, mientras que el desarrollo de la periodontitis en los ratones PGPD *Mif-/-* es menos severa y producen menos citocinas proinflamatorias como TNF-α e IFN-γ y menor expresión de la metaloproteinasa (MMP)-2 y MMP-13 con respecto a los ratones PGPD WT.

Nuestros resultados sugieren que MIF es una citocina responsable de forma parcial de la inflamación asociada a la severidad de la periodontitis, y es esencial en la exacerbación de la periodontitis durante la gestación, principalmente cuando esta patología se desarrolla previa a la gestación.

Abstract

Periodontal disease (PD) is an inflammatory alteration of the periodontal tissues of heterogeneous ethology that affects the gingiva, periodontal ligament, root cementum and alveolar bone, with the consequent loss of clinical attachment of the dental organs. PD is one of the most common diseases; approximately 60% of the world's population has some degree of this disease. This condition affects more than 23% of women between the ages of 23 and 54 and has been reported to be present in 56% of pregnant women.

On the other hand, macrophages migration inhibitory factor (MIF) is a proinflammatory cytokine that is significantly elevated in periodontitis, as well as at the beginning and end of pregnancy. Although periodontitis usually occurs with greater severity during pregnancy, the participation of MIF in the development and severity of periodontitis during pregnancy has not been established. To delve deeper into the participation of MIF in the exacerbation of PD during pregnancy, in this study a murine model of PD was developed. We used WT and *Mif−/−* mice on BALB/c genetic background. To induce periodontitis (PT), a 6-0 nylon suture was placed around the upper right second molar. To generate pregestational periodontal disease (PGPD), PT was induced two weeks prior to the mate. We evaluated histological changes, performed histometric analysis of clinical attachment loss (CAL), expression and activation of MMP-2 and MMP-13 by immunofluorescence and zymogram, as well as concentration of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-6 and IL -10 in serum by ELISA and the in situ relative expression of *mif, tnf-α, ifn-γ* and *il-17* by qPCR.

Our results show that the periodontal tissues of PGPD WT mice produce 2 times more MIF, while the development of periodontitis in PGPD *Mif-/-* mice is less severe and they produce less proinflammatory cytokines such as TNF-α and IFN-γ and lower expression of metalloproteinase (MMP)-2 and MMP-13 compared to PGPD WT mice.

Our results suggest that MIF is a cytokine partially responsible for the inflammation associated with the severity of periodontitis and is essential in the exacerbation of periodontitis during pregnancy, mainly when this pathology develops prior to pregnancy.

1 Introducción

La enfermedad periodontal (EP) es una alteración inflamatoria de los tejidos periodontales de etología heterogénea que afecta a los tejidos periodontales: encía, ligamento periodontal, cemento radicular y hueso alveolar, según la nueva la nueva clasificación de enfermedades periodontales, la gingivitis, afecta únicamente a la encía, mientras que la periodontitis (PT), afecta a todos los tejidos periodontales y provoca principalmente la destrucción de hueso alveolar, con la consecuente pérdida de inserción clínica de los órganos dentarios (1, 2). Es una de las enfermedades más comunes (3), en 2017 aproximadamente el 60% de la población a nivel mundial presentaba algún grado de enfermedad periodontal (4, 5), y en Latinoamérica el porcentaje reportado en 2016 fue de hasta el 90% (6). En particular la gingivitis tiene una prevalencia de entre el 50% y el 94% en adultos (7), mientras que, en México para el 2018, alcanzaba el 96.6% (8).

Para el desarrollo de la enfermedad periodontal contribuyen diferentes factores, principalmente en la interacción entre la respuesta inmune del hospedero hacia el biofilm, y esta respuesta puede ser modificada por predisposición genética y epigenética, factores sociales, hábitos como fumar, uso de alcohol y mala higiene oral (9), envejecimiento (10), enfermedades sistémicas como obesidad, desnutrición (11), infecciones, diabetes tipo 1 y 2.

De manera particular, la periodontitis en 2014 afectaba a más de 23% de mujeres en edades entre los 23 y los 54 años, y se ha reportado su presencia en el 56% de las mujeres gestantes (12). La gingivitis y periodontitis preexistentes pueden progresar durante la gestación, algunos estudios muestran que algunos parámetros como índice de placa, profundidad al sondeo, sangrado gingival se deterioran durante la gestación, aunque presenten índices de placa similares comparados con mujeres no gestantes (13). La gestación es una condición que modifica el perfil hormonal, pero poco se ha descrito sobre sus efectos sobre el desarrollo de la periodontitis, aunque la interacción entre la concentración de hormonas gestacionales y la enfermedad periodontal parece ser bidireccional (14), durante la gestación las hormonas sexuales favorecen respuestas alteradas de las células inmunes, así como la modificación de la microbioma lo que favorece el desarrollo de la enfermedad

periodontal. (Anexo II: Ortiz-Sánchez BJ, Legorreta-Herrera M, Rodriguez-Sosa M. Influence of Gestational Hormones on the Bacteria-Induced Cytokine Response in Periodontitis. Mediators Inflamm. 2021 Oct 18;2021:5834608. doi: 10.1155/2021/5834608. PMID: 34707462; PMCID: PMC8545568) (14).

2 Objetivos

2.1 Objetivo General:

Identificar la participación del factor inhibidor de la migración de macrófagos (MIF) en la exacerbación de la periodontitis experimental, previo y durante la gestación en un modelo murino

2.2 Objetivos Particulares:

Para alcanzar el objetivo general se desarrollaron los siguientes objetivos particulares en ratones hembra WT y *Mif-/-*, con 4 grupos experimentales: periodontitis pregestacional (PGPD), periodontitis (PT), gestantes (P) y control (CTL).

1. Determinar si la ausencia de MIF influye en la severidad de la periodontitis pregestacional.
2. Identificar el daño tisular clínico e histológico a través de la medición de la perdida clínica de inserción periodontal por el desarrollo de la periodontitis experimental inducida previa a la gestación en ausencia de MIF en los diferentes grupos experimentales.
3. Identificar las diferencias en la distribución del colágeno por tinción de Masson en los diferentes grupos experimentales.
4. Identificar la expresión local de MMP-2 y MMP-13, por inmunofluorescencia en los diferentes grupos experimentales.
5. Cuantificar *in situ* los transcritos de citocinas proinflamatorias *mif, tnf-α, ifn-γ* e *il-17* por qPCR en los diferentes grupos experimentales.
6. Determinar la actividad enzimática de MMP-2 y MMP-13 por zimograma en los diferentes grupos experimentales.
7. Determinar las concentraciones de citocinas IFN–γ, TNF-α, IL-6, IL-10 en suero por ELISA en los diferentes grupos experimentales

3 Antecedentes

3.1 Microbioma Oral en la enfermedad periodontal y la gestación

Uno de los factores preponderantes para el desarrollo de la enfermedad periodontal es el microbioma (15), el cual se organiza en comunidades microbianas que forman biofilms, estos se encuentran embebidos en matriz extracelular y exopolímeros, los cuales se adhieren a las superficies mucosas y dentales de la cavidad oral (16). Las interacciones entre especies microbianas pueden modificar el sinergismo microbiano, que cuando se altera el equilibrio y se encuentra en disbiosis promueve el desarrollo de la enfermedad periodontal (17).

El microbioma subgingival relacionado con la inducción de la enfermedad periodontal fue revisado en el artículo de revisión, Ortiz-Sánchez BJ, Legorreta-Herrera M, Rodriguez-Sosa M. Influence of Gestational Hormones on the Bacteria-Induced Cytokine Response in Periodontitis. Mediators Inflamm. 2021 Oct 18;2021:5834608. doi: 10.1155/2021/5834608. PMID: 34707462; PMCID: PMC8545568 (14).

Sin embargo, durante la gestación el microbioma se encuentra en disbiosis y se vuelve patógeno, lo cual se puede revertir durante el periodo de posparto. La mayoría de los estudios mencionan abundancia de anaerobios como *Prevotella intermedia, Porphyromonas gingivalis, Tannerella forsythia, Campylobacter rectus, Fusobacterium nucleatum y Actinobacillus actinomycetemcomitans* (18), la presencia de *Prevotella intermedia* es preponderante debido a que su desarrollo depende de la interacción con las hormonas sexuales (19), sin embargo, Balan en 2018, identificó de las 20 especies en la gingivitis gestacional, solamente *Porhyromonas endodontalis* y *Fretibacterium sp OT 361* en una abundancia del 0.5% en saliva de mujeres gestantes (13).

Por otra parte, se ha reportado un cambio en la concentración de bacterias anaeróbicas a aeróbicas en el segundo trimestre de gestación, principalmente las concentraciones de *Bacteroides intermedius,* relacionadas con las concentraciones plasmáticas de estrógenos y progesterona (20, 21). Recientemente se ha descrito una respuesta inmune dispar a *Porphyromonas gingivalis* en ratones gestantes con infección periodontal los cuales

mostraron una disminución en la destrucción de los tejidos periodontales (22) o un agravamiento debido a un desbalance en la relación entre las células T colaboradoras (helper) - 17 (Th-17)/ Células T reguladoras (Treg) en un modelo murino de periodontitis inducido con *Porphyromonas gingivalis* (23).

La relación entre la enfermedad periodontal y la gestación ha sido controversial; por un lado, autores como González-Jaranay (24) han identificado en mujeres gestantes algún grado de enfermedad periodontal, además de la progresión y agravamiento de los síntomas a lo largo de la gestación, sin embargo al terminar la gestación, en el posparto, los datos clínicos mejoran. Por otra parte, diversos autores como Martínez-Martínez(25), mencionan que la enfermedad periodontal materna no es un factor de riesgo si se controlan factores como los procesos infecciosos (25, 26). Sin embargo, otros autores no encontraron evidencia contundente de la interacción de la enfermedad periodontal y la gestación, sin embargo, recomiendan la terapia periodontal de rutina en mujeres gestantes como tratamiento seguro para las madres y los fetos, además favorece la mejoría en los signos clínicos de la enfermedad periodontal materna (27, 28). Por el contrario, también se ha descrito evidencia de un fuerte vínculo entre la enfermedad periodontal y alteraciones en el desarrollo de la gestación como preeclamsia, parto pretérmino y bajo peso del producto al nacer, atribuibles a la enfermedad periodontal (29-31), así como asociaciones con alteraciones metabólicas como la obesidad y la diabetes (32, 33).

3.2 Respuesta inmune durante la enfermedad periodontal

Las bacterias presentan patrones moleculares asociados a patógenos (PAMPs) como lipopolisacáridos (LPS) y ácido lipoteicoico, así como enzimas como colagenasa, proteinasa, hialuronidasa, leucotoxinas y metabolitos, los cuales pueden destruir los tejidos periodontales y liberar mediadores inflamatorios y patrones moleculares de daño (DAMPs) y activar la respuesta inmune del hospedero (34).

La interacción de los PAMPS con el sistema inmune del hospedero se realiza a través de receptores de reconocimiento de patrones (PRRs) como los receptores tipo Toll (TLR), los cuales se expresan en las células del sistema inmune, este reconocimiento permite la

activación de la respuesta inmune innata. En particular, los TLR presentes en las células al reconocer a los PAMPs llevan a la activación de varios factores de transcripción como al factor nuclear κB (NFκB), o el factor activador de la proteína 1 (AP-1) a través de la cascada de la proteína quinasa activada por mitógenos (MAPK) (35).

Las células inmunes y los tejidos periodontales expresan TLRs: el epitelio gingival TLR2, 3, 4, 5, 6; los fibroblastos gingivales TLR2, 4, 9; los fibroblastos del ligamento periodontal TLR 2, 4; los osteoblastos TLR1, 4, 5, 6, 9; los osteoclastos TLR1, 2, 3, 4, 5, 6, 7, 8; el cemento TLR2, 4; los neutrófilos TLR1, 2, 4, 5, 6, 7, 8, 9, 10 y las células de Langerhans/ células dendríticas (DCs) tisulares TLR1, 2, 3, 4, 5, 6, 8, 10 (36) figura 1.

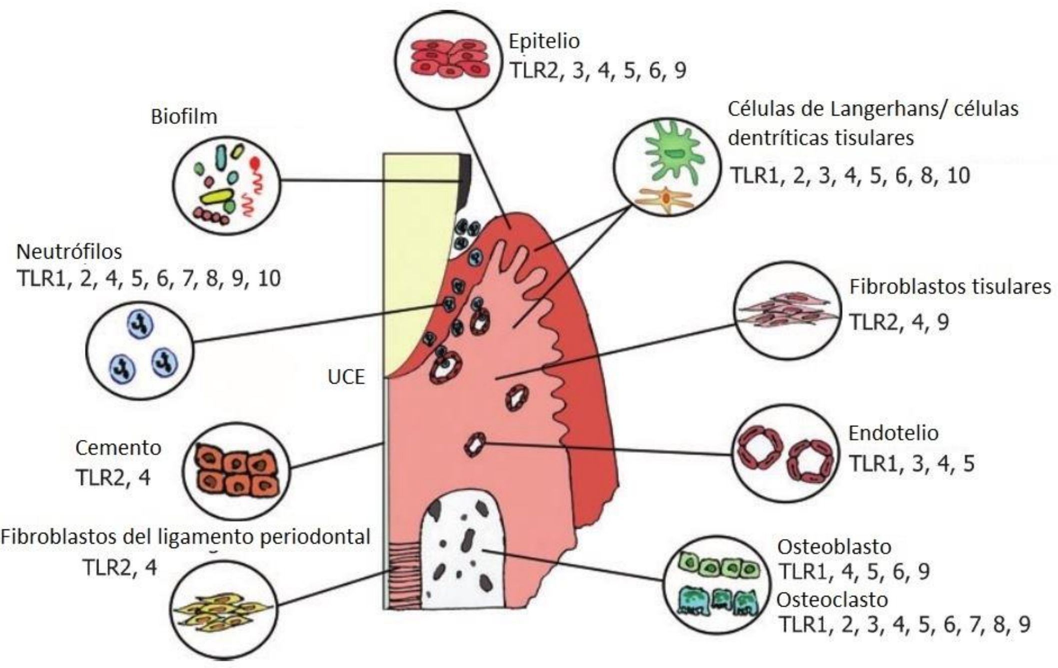


Fig. 1. Expresión de RNA de receptores tipo Toll (TLRs) en diferentes células de tejidos periodontales (Modificada de Mahanonda, 2007)(36)

La interacción del microbioma con las células del sistema inmune: neutrófilos, monocitos/macrófagos (Mϕ), células dendríticas (DCs), células T, linfocitos B y células plasmáticas favorece la activación del sistema inmune. En la enfermedad periodontal se presenta fases agudas y cónicas por lo que se expresan de manera alternada la respuesta

inmune inflamatoria, caracterizada por la presencia de interleucina (IL)-1α, IL-1β, IL-8, factor de necrosis tumoral α (TNF-α) e interferón γ (IFN-γ), y la respuesta antinflamatoria, representada por la presencia de las citocinas IL-4, IL-13, y las citocinas reguladoras IL-6 e IL-10. La expresión de citocinas en la enfermedad periodontal varía entre los tejidos, de manera que las citocinas inflamatorias se han detectado en tejidos gingivales inflamados (37); y las citocinas antinflamatorias se han encontrado en tejidos con enfermedad periodontal (38, 39).

Se ha reportado que las citocinas proinflamatorias que exacerban la inflamación en la enfermedad periodontal son IL-1β, TNF-α e IL-17, y sinérgicamente favorecen la expresión de IL-6. En los fibroblastos gingivales se incrementa la producción de IL-1β y TNF-α, metaloproteinasa (MMP) -1 y MMP-3 y en menor grado IL-17 (40). Por su parte, IL-17 e IFN-γ favorecen la expresión de la enzima indoleamina 2,3-dioxigenase (IDO) que tiene efectos de supresión sobre la respuesta inmune en cultivos de fibroblastos gingivales (41). La expresión de mediadores inflamatorios en tejidos periodontales, favorecen la expresión de IL-17 (42). Además, los niveles elevados de IL-17, factor de crecimiento transformante β (TGF-β), IL-1β e IL-6 en presencia de células Th-17, favorecen la expresión de receptor activator of NF-κB ligand (RANKL) en hueso alveolar en periodontitis (43). Adicionalmente, estudios recientes sugieren que bacterias periodontopatógenas como *Porphyromonas gingivalis* favorecen la disbiosis al aumentar la expresión de IL-17-A e IFN-γ lo que favorece la conversión de fase aguda a cónica (44).

3.3 Hormonas sexuales y la enfermedad periodontal

Con respecto a la interacción de las hormonas sexuales y el desarrollo de la enfermedad periodontal, se sabe que las hormonas sexuales modulan la respuesta inmune y participan en procesos como la maduración, la selección de células inmunes, el tráfico, la expresión de moléculas de histocompatibilidad, proliferación celular y la producción de citocinas. De manera importante, evidencias recientes indican que las hormonas gestacionales como el 17β estradiol, estriol y progesterona influyen el desarrollo de la periodontitis, ver la revisión producto del Doctorado (Anexo II, Ortiz-Sánchez BJ, Legorreta-Herrera M, Rodriguez-Sosa

M. Influence of Gestational Hormones on the Bacteria-Induced Cytokine Response in Periodontitis. Mediators Inflamm. 2021 Oct 18;2021:5834608. Doi: 0.1155/2021/5834608. PMID: 34707462; PMCID: PMC8545568.)

Las hormonas sexuales femeninas alteran la respuesta de los tejidos periodontales al microbioma. Durante la gestación los tejidos gingivales presentan una respuesta exagerada a los irritantes, de manera que los rangos de inflamación presentes en esta población fluctúan de edema ligero y enrojecimiento a inflamación severa con sangrado e hiperplasia gingival. Por otro lado, la carga hormonal de mujeres usuarias de anticonceptivos orales (AO's) difiere de la condición hormonal de la gestación, y favorece en mayor cantidad el exudado en los tejidos gingivales inflamados (45). Las hormonas sexuales también pueden favorecer cambios en los microorganismos presentes en los grupos de mujeres gestantes y usuarias de AO's comparadas con grupos de mujeres no gestantes (46), lo que ha sugerido que la carga hormonal contribuye al desarrollo de la enfermedad periodontal (47).

3.4 Factor inhibidor de la migración de macrófagos (MIF) y la enfermedad periodontal

Uno de los moduladores de la respuesta inmune inflamatoria es el factor de inhibición de migración de macrófagos (MIF) (48), el cual inicialmente se detectó como una secreción de linfocitos T activados y en fibroblastos, es conocido como el principal contrarregulador de glucocorticoides (GCs) (49). MIF se une a CD74, la cadena invariante del complejo principal de histocompatibilidad clase II, en células inmunes como los macrófagos y las células dendríticas para desencadenar una respuesta inmune inflamatoria (TNF-α e IL-6) (50), además de que favorece la expresión del receptor TLR-4 (51) y está involucrado en la reparación de tejidos (52). MIF también se ha asociado a procesos tales como la ovulación y ciclo menstrual, así mismo, se ha descrito una alta expresión de MIF durante el embarazo temprano y el embarazo a término, incremento que se acentúa durante el parto (53, 54).

Se ha sugerido que MIF tiene importante participación en la fisiopatología del tejido gingival, ya que se expresa en tejidos periodontales, principalmente en encía libre y en el epitelio de unión, así como en queratinocitos, células basales y fibroblastos (55). Se ha reportado aumento en la concentración de MIF en fluido crevicular, saliva y suero de

pacientes con periodontitis crónica (sanos 8.85±1.28 ng/mL, 31.5±5.76ng/mL, and 7.08±0.58 ng/mL vs pacientes con periodontitis 17.85±1.04 ng/mL, 72.94±10.90 ng/mL, and 9.91±1.65 ng/mL) (56); en pacientes con periodontitis en estadios III y IV también se encuentran concentraciones de MIF elevadas en suero (57, 58). También se ha sugerido que MIF tiene un papel importante en la enfermedad periodontal y en su patogénesis (59) favoreciendo, además de inflamación, la osteoclastogénesis (60). Evidencias recientes demuestran que MIF favorece la expresión de las MMPs en la periodontitis, así como en artritis reumatoide (61).

La presencia de MIF también se ha reportado en los tejidos periodontales sanos, con una expresión abundante en el líquido crevicular con una correlación positiva con las concentraciones de neutrófilos. Se ha propuesto que la expresión constitutiva de MIF por las células epiteliales puede ser explicada por la exposición de la encía a los estímulos externos y a la homeostasis (62).

3.5 Metaloproteinasas (MMPs) en la enfermedad periodontal

Las MMPs son una familia de endopeptidasas neutras dependientes de zinc y calcio presentes en el desarrollo, la regeneración y la enfermedad periodontal. Las MMPs están encargadas de degradar las proteínas integrantes de la matiz extracelular (EMC) (63). En periodonto se reconocen: las colagenasas MMP-1, MMP-8 y MMP-13, gelatinasas (MMP-2 y MMP-9) y las estromelinasas (MMP-3 y MMP-10) (64-66).

Se ha reportado la participación de las MMP’s en la degradación de matriz extracelular en la enfermedad periodontal, entre ellas MMP-13 y la MMP-2. Particularmente, la MMP-13, tanto en su forma de pro-enzima (~60 kDa) como en su forma activa (~45-50 kDa), degradan colágeno I, II, III, membranas basales de colágeno IV, proteoglicanos, fibronectina, fibrina y tensina. MMP-13 se expresa en el epitelio del surco y en los fibroblastos gingivales (67). Las MMP-2 y MMP-9, pueden degradar colágeno tipo IV, V, VII, X, XI Y XII, fibronectina y elastina, y en medio ácido pueden degradar colágeno tipo I. La pro-enzima de MMP-2 (~72 kDa), secretada por fibroblastos, células endoteliales y osteoblastos, su forma activa (~59-63 kDa) ambas participan principalmente en la degradación de elastina (68, 69).

Patógenos periodontales como *Actinobacillus gingivalis* y *Phorphiromonas gingivalis* favorecen, la activación y sobreexpresión de las MMPs (67). Es ampliamente reconocido que la degradación de colágeno tipo I, II y III por MMP-1, MMP-8, MMP-13, MMP-18 desencadenan la destrucción de la matriz extracelular (70).

MIF es una citocina que se ha reportado sobreexpresada en tejidos periodontales y en fluido crevicular (71), así como en tejidos gestacionales durante la gestación (54, 72), se ha propuesto a MIF como una citocina que participa en el desarrollo de la enfermedad periodontal. Abordamos la influencia de MIF en la severidad de la periodontitis pregestacional, en un principio nos enfocamos en identificar los cambios clínicos e histológicos de los diferentes grupos experimentales, así como en la concentración de citocinas en suero de los diferentes grupos experimentales.

4 Metodología

Para poder resolver los objetivos planteados se desarrolló un modelo murino de periodontitis experimental en ratones hembra WT y *Mif-/-,* el protocolo completo se encuentra en el artículo requisito (Anexo I. Ortiz-Sánchez BJ, et. al. Periodontitis exacerbation during pregnancy in mice: Role of macrophage migration inhibitory factor as a key inductor. J Periodontal Res. 2023 Nov 21. doi: 10.1111/jre.13211. Epub ahead of print. PMID: 37990413) (73). La descripción completa de las técnicas utilizadas en el presente trabajo se agrega como anexo III.

Brevemente, basados en un protocolo previamente reportado (74, 75), se colocó una sutura de nylon 6-0 alrededor del segundo molar superior derecho, para inducir enfermedad periodontal (PD). Dos semanas posteriores a la inducción la mitad de los ratones fueron separados para la cruza, para establecer 4 grupos experimentales: 1) sanos (CTL) ratones de la misma edad sin periodontitis o gestación, 2) gestantes (P) (3 semanas de gestación + 4 semanas hasta el destete), 3) periodontitis (PT) ratones a los que se les indujo periodontitis, pero no gestantes y 4) periodontitis previa a gestación (PGPD) a los que se les indujo PT 2 semanas previas a la cuza. Después de nueve semanas postinducción, cuatro semanas después del destete, los ratones fueron eutanizados. Con los tejidos se realizaron:

Histología: Se realizó tinción con hematoxilina y eosina (H&E) en secciones coronales del segundo molar derecho para observar los tejidos periodontales y medir la pérdida de inserción clínica (CAL) y los resultados se compararon entre los grupos de control y experimental.

Los cortes histológicos teñidos fueron observados en un microscopio óptico (Carl Zeiss Axio Vert. A, Berlin, Germany), para hacer un análisis descriptivo de los cambios en configuración del epitelio del surco, fibras gingivales, fibras periodontales (principalmente fibras crestales), altura e integridad de la cresta alveolar.

La profundidad del nivel de inserción clínica (Clinical Attachment Level, -CAL-) se determinó considerando la distancia entre la unión cemento–esmalte (UCE) y el punto más

cercano de la cresta alveolar, se realizó un análisis histométrico, empleando el programa ZEN 2 Blue edition (Carl Zeiss Microscopy GmbH, 2011), trazando una línea entre ambos puntos, en las caras vestibular y palatina, basado en lo reportado por Semenoff (76).

Inmunofluorescencia: La expresión relativa de MMP-2 y MMP-13 fue medida con la técnica de inmunofluorescencia indirecta (RIF). Las laminillas se analizaron con el microscopio Confocal (Leica, SP2, Wetzlar, Germany), se observaron en la región del epitelio de unión a 60X, en esa región se seleccionaron 20 áreas (ROIs) por laminilla, para determinar la intensidad de fluorescencia relativa.

PCR cuantitativa (qPCR). RNA total fue extraído de los maxilares derechos, utilizando QIAcube and RNeasy Mini Kit (Qiagen, Venlo, Netherlands), El DNA complementario (cDNA) se obtuvo con RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), y la qPCR con PowerSYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Los valores relativos de expresión génica se normalizaron con respecto a la expresión constitutiva de la β-actina. Los cebadores específicos para los genes diana se muestran en la tabla 1 del artículo (todos sintetizados por Sigma-Aldrich) (73)

Zimograma: Para identificar la actividad de las MMPs, se realizó un zimograma en gel de acrilamida con sustrato de gelatina (Sigma Aldrich) para MMP-2, y colágena de piel de ternera (Sigma Aldrich) para MMP-13, para lo cual se extrajeron proteínas de los maxilares derechos, para identificar la presencia y actividad de MMP-2 pro-enzima (~72 kDa) y su forma activa (~59-63 kDa), y de MMP-13 pro-enzima (~ 57-60 kDa) y su forma activa (~56-45 kDa)**.**

ELISA: Se determinó la concentración de citocinas por medio de ELISA-sándwich en los sueros obtenidos de los ratones de los grupos experimentales (ELISA Development Kit para TNF-α (900-K54), IL-10 (900-K53), IL-6 (900-K50), IFN-γ (900-K98), Peprotech, New Jersey, USA)

5 Análisis estadístico

El análisis de los datos obtenidos entre los diferentes grupos WT y Mif-/-, se llevaron a cabo mediante la prueba de ANOVA no paramétrica, seguida de la prueba de Tukey de comparaciones múltiples, y la U de Mann-Whitney, Valores con $p<0.05$ considerados como estadísticamente significativos: (a) comparados con los ratones sanos (CTL), (b) comparados con el grupo gestante (G), (c) comparado con el grupo de periodontitis (PT), (*) WT comparado con *Mif-/-*. Utilizando el programa GraphPad Prism 8.0 (Graph Pad Software, Inc., San Diego CA, USA).

6 Justificación

Los antecedentes relacionados con la influencia de MIF en el desarrollo de la enfermedad periodontal y la sobreexpresión de MIF durante la gestación, el parto y posparto, sugieren que la presencia de MIF podría influir en el desarrollo de la periodontitis inducida previa a la gestación, y esta verse exacerbada durante la gestación.

MIF, una citocina esencial en el desarrollo de la respuesta inflamatoria presenta cambios dinámicos de expresión durante los diferentes estadios de la gestación, pero hasta ahora no se ha establecido la influencia de MIF presente en la gestación y el desarrollo de la periodontitis observada en esta población.

Por lo tanto, éste estudio contribuirá a identificar la relación de MIF en el desarrollo de la periodontitis experimental inducida previa a la gestación l.

7 Hipótesis

MIF participa en el desarrollo y exacerbación de la enfermedad periodontal durante la gestación, favorece la destrucción y remodelación tisular, favorece la expresión de metaloproteinasas y mediadores inflamatorios en un modelo murino de periodontitis previa a la gestación.

8 Resultados

La mayoría de los resultados fueron publicados en el artículo requisito (Anexo i): "Periodontitis exacerbation during pregnancy in mice: role of macrophage migration inhibitory factor (MIF) as a key inductor". J Periodontal Res. 2023 Nov 21. doi: 10.1111/jre.13211. Epub ahead of print. PMID: 37990413) (73).

8.1 Los tejidos periodontales de los ratones con periodontitis pregestacional expresaron más MIF en comparación con los ratones con periodontitis

Al establecer las concentraciones de MIF en los tejidos periodontales, identificamos una sobreexpresión significativa de MIF en los tejidos periodontales de los grupos P y PGPD, en comparación con el grupo control, mientras que no se identificaron transcritos en los grupos *Mif-/-* (73) (Fig 2, Anexo I).

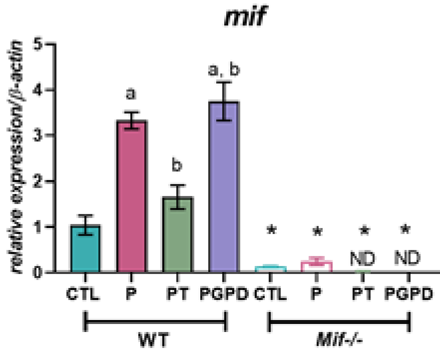


Fig 2. La periodontitis induce la expresión de MIF y el embarazo induce la sobreexpresión del MIF en la enfermedad periodontal previa a la gestación. Se eutanizaron ratones hembra con periodontitis (PT) y ratones hembra con periodontitis pregestacional (PGPD) 9 semanas después de la inducción de periodontitis; la expresión de MIF en tejido periodontal se determinó mediante RT-qPCR. Se utilizaron como controles ratones sanos (CTL) y gestantes (P). Los datos se expresan media ± SEM y son representativos de dos experimentos independientes con al menos tres ratones por grupo. Los valores de $p<0.05$ se consideraron estadísticamente significativos (a) comparados con ratones sanos (CTL), (b) comparados con el grupo de gestantes (P) y (c) comparados con el grupo de periodontitis (PT), (*) *Mif-/-* vs WT, (ND) no detectado, utilizando ANOVA con prueba de comparación múltiple de Tukey y prueba U de Mann-Whitney.

8.2 Los ratones Mif-/- desarrollaron signos clínicos atenuados en periodontitis pregestacional

Al observar las características clínicas de los tejidos de los diferentes grupos, observamos los signos de la enfermedad periodontal, inflamación severa, enrojecimiento, alrededor del primer molar en el grupo WT PT, mientras que los grupos Mif-/- no mostraron cambios clínicos inflamatorios (73) (Fig 3, Anexo I).

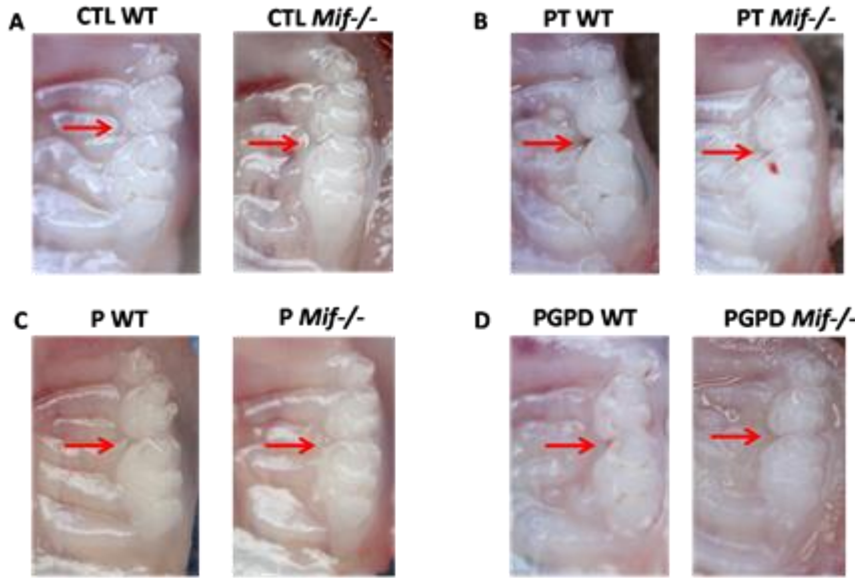


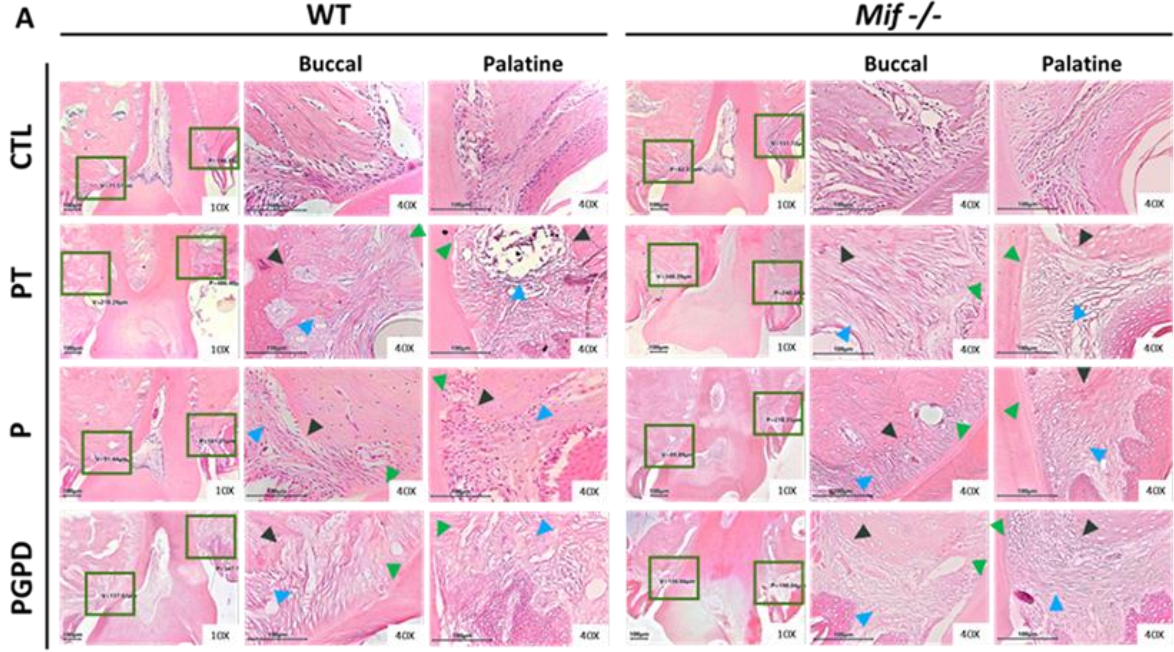
Fig. 3. Los ratones *Mif-/-* desarrollan síntomas clínicos atenuados en la enfermedad periodontal pregestacional. Fotografías clínicas de tejidos periodontales en los grupos A. CTL WT y *Mif-/-*, B. PT WT y Mif-/-, C. P WT y *Mif-/-*, D, PGDP WT y *Mif-/-*, después de la inducción de periodontitis (PT), alrededor del segundo molar superior derecho. La flecha roja indica la región interproximal y la encía palatina. Las imágenes son representativas de tres experimentos independientes con n= 3.

8.3 La deleción de Mif disminuye el daño de tejidos periodontales y reduce la pérdida de inserción clínica (CAL) en periodontitis pregestacional

Las características clínicas nos permitieron evaluar la ausencia de cambios infamatorios en los tejidos periodontales de los grupos *Mif-/-* con respecto a los grupos WT. Se identificó que la mayor destrucción tisular con pérdida de inserción se presentó en los grupos PT WT y PGPD WT, y la mayor destrucción ocurrió en la cara palatina. Este primer acercamiento,

nos permitió identificar cambios en la configuración de los tejidos blandos, encía y ligamento periodontal (Fig 4A, Anexo I)

La confirmación de los datos clínicos e histológicos se realizó a través de un análisis histométrico, en el cual se midió el CAL en los diferentes grupos experimentales, los grupos que presentaron la mayor destrucción del tejido y la mayor profundidad de CAL fueron PT WT y PGPD WT tanto en la cara palatina, como en la cara vestibular, en contraste con los grupos P y CT, mientras que en los grupos *Mif-/-,* no se observaron cambios con respecto a las características clínicas del tejido y la profundidad del CAL. Cabe mencionar que la pared palatina presentó una mayor pérdida de integridad del tejido óseo, contrario a lo que sucede en humanos en quienes la pared vestibular es más delgada y en ocasiones inexistente (73) (Fig 4 B, C, Anexo I).



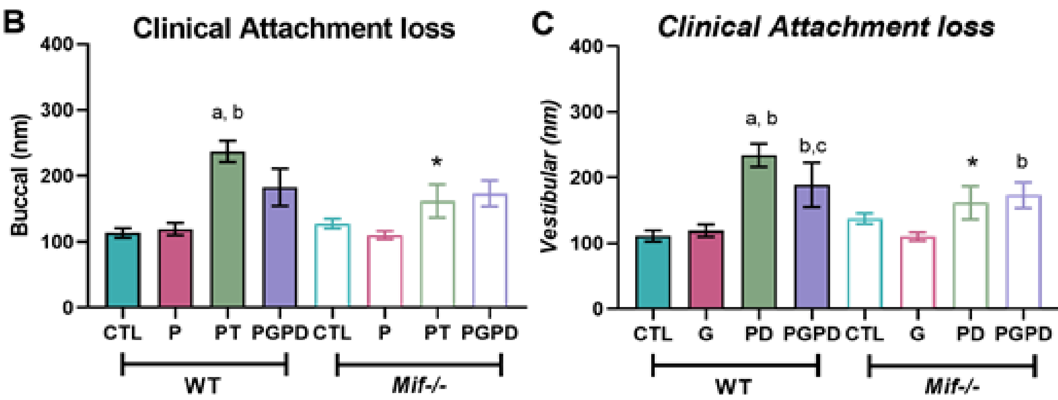


Fig. 4. Los ratones *Mif-/-* muestran menor daño al tejido periodontal y pérdida de inserción que los ratones wild-type en el modelo de periodontitis pregestacional. A) Secciones histológicas representativas teñidas con H&E, se muestra la medición de CAL, trazando una línea desde la UEC hasta la cresta ósea. Primer panel 10X, marcado en el panel verde del cuadro 40X, en las superficies bucal/vestibular izquierda (V) y palatina derecha (P), WT vs *Mif-/-*. Puntas de flecha: pérdida de la cresta alveolar (gris), migración del epitelio del surco (azul), inserción de fibras de Sharpey (verde). Análisis histométrico de la pérdida de inserción clínica. B) Bucal, C) Palatino. Grupos CTL WT, PT WT, P WT y PGPD WT, CTL *Mif-/-*, PT *Mif-/-*, G *Mif-/-* y PGPD *Mif-/-*. Se muestra una imagen representativa de tres experimentos independientes. Se consideraron estadísticamente significativos valores de $p<0.05$, (a) comparados con ratones sanos (CTL), (b) comparados con el grupo gestante (P), y (c) comparados con el grupo de periodontitis (PT), (*) *Mif-/-* vs WT, utilizando ANOVA con prueba de comparación múltiple de Tukey y prueba U de Mann-Whitney.

8.4 Los ratones Mif-/- muestras reorganización de las fibras de colágena

Este primer acercamiento, nos permitió observar cambios en la configuración de los tejidos blandos, encía y ligamento periodontal, lo que nos llevó a identificar, a través de la tinción de Masson, la reconfiguración y engrosamiento en el colágeno en los grupos PT *Mif-/-* y PPG *Mif-/-*, que mostraron una mayor proporción de colágeno en el PDL y en las fibras de Sharpey que el grupo P WT, y de manera muy interesante, el grupo PGPD *Mif-/-* presentó fibras de Sharpey ensanchadas, con colágeno denso, firmemente adheridas al cemento radicular. Este hallazgo a nuestro conocimiento fue reportado por primera vez (73) (Fig. 5, Anexo I).

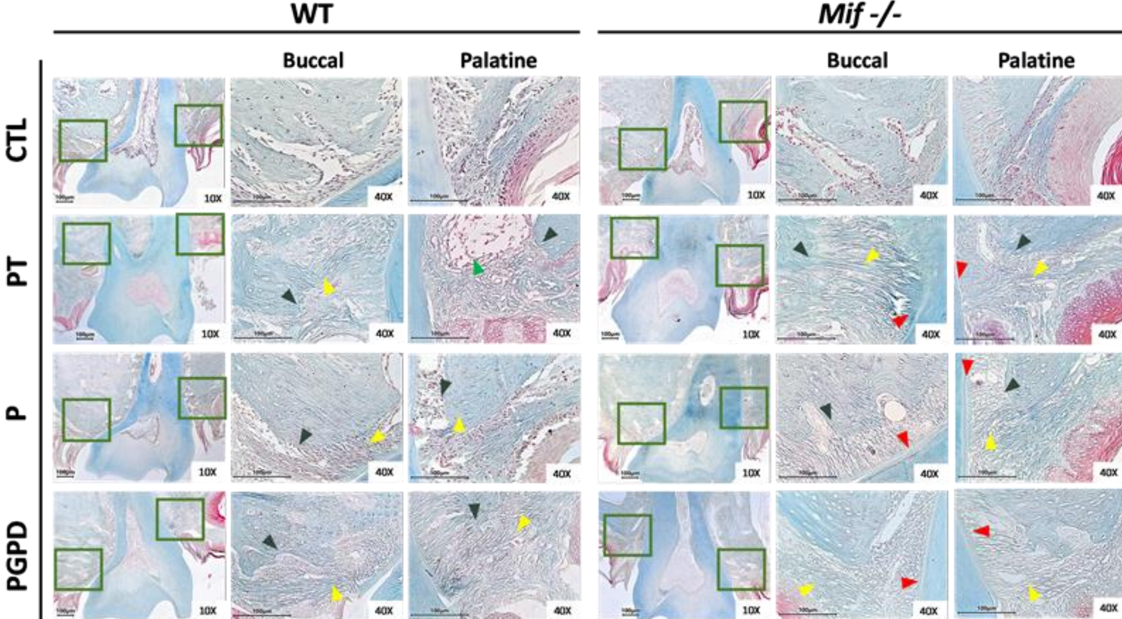
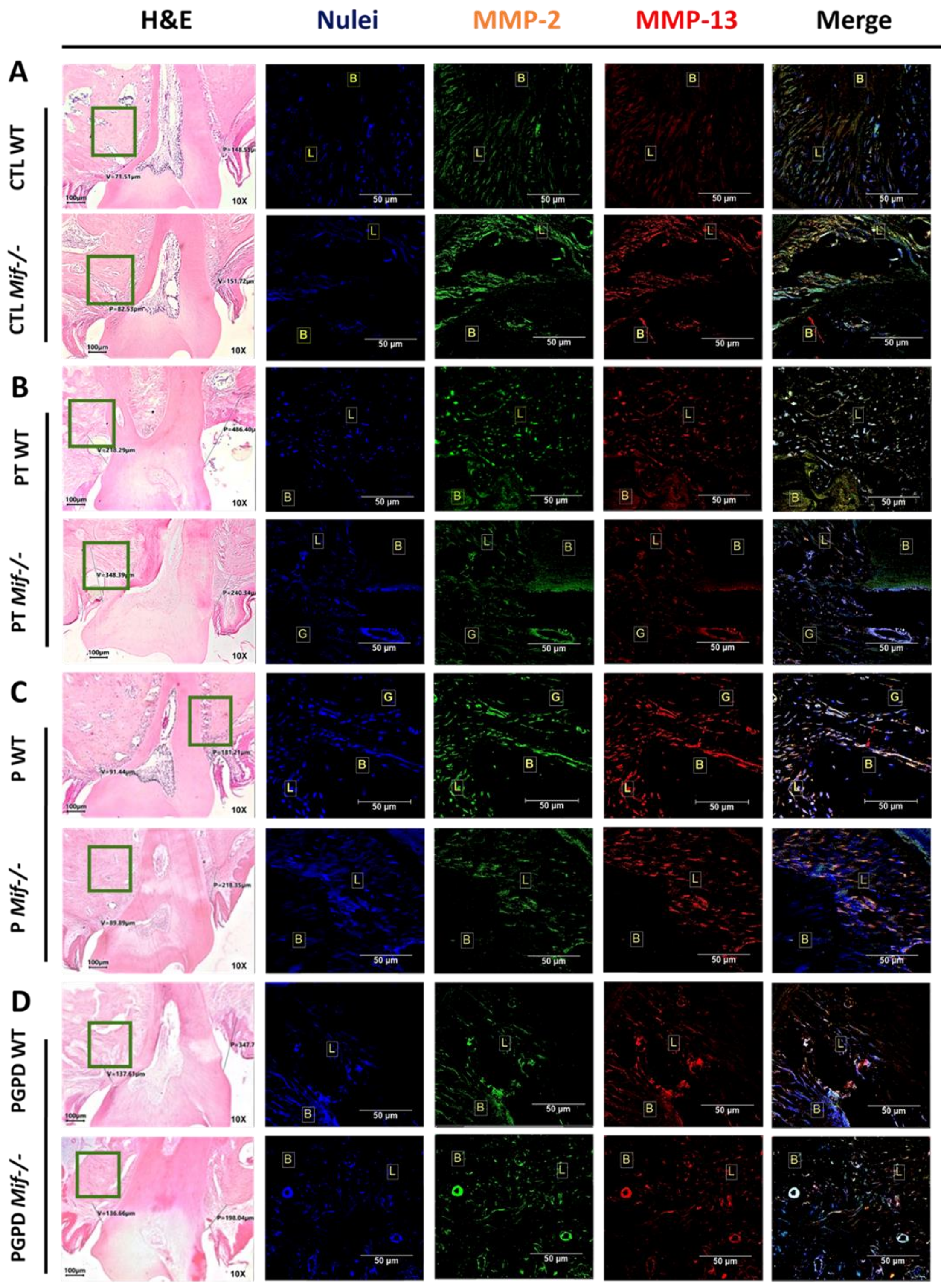


Fig. 5. La deleción del MIF favorece la conservación del colágeno y del epitelio de la bolsa periodontal. Secciones histológicas representativas teñidas con tricrómico de Masson, se muestra la distribución del colágeno en los tejidos periodontales. Primer panel 10X, marcado en el cuadro verde 40X, en las superficies bucal/vestibular izquierda (V) y palatina derecha (P), WT vs *Mif-/-*. Para identificar colágeno (azul), queratina (rojo) y núcleos (negro), queratina y epitelio (rojo), puntas de flecha: pérdida de cresta alveolar (gris), migración de queratina (verde), agrandamiento de colágeno (rojo), distribución de fibras gingivales (amarillo). Grupos CTL WT, PT WT, P WT y PGPD WT, CTL *Mif-/-*, PT *Mif-/-*, P *Mif-/-* y PGPD *Mif-/-*. Se muestra una imagen representativa de tres experimentos independientes, n=6.

8.5 La periodontitis pregestacional inducida en ratones Mif-/- muestran disminución en la expresión de MMP-2 y MMMP-13 durante la gestación

Los cambios en la distribución y reconfiguración de las fibras de colágeno en los tejidos periodontales de los grupos con PT, nos hizo preguntarnos si MMP-13 (colagenasa) y MMP-2 (gelatinasa) presentaban cambios en la fluorescencia relativa, los resultados reforzaron las observaciones sobre la influencia de MIF en la expresión de MMP-2 asociada a la progresión de la PT, ya que la RFI de MMP-2, fue mayor en los grupos P WT y PT WT, mientras que en los grupos deficientes de MIF, únicamente identificamos aumento en la RFI en el grupo PT *Mif-/-*, sin embargo, dicha expresión fue significativamente menor que el grupo PT WT. (Fig 6E, Anexo I) En cuanto a MMP-13, los grupos P WT y PGPD WT presentaron aumentó en la RFI, mientras que para los grupos *Mif-/-*, solo P *Mif-/-* presento

un leve aumento con respecto a su control, destaca que la expresión basal de MMP-13 fue ligeramente mayor en el grupo CTL WT (73) (Fig 6F, Anexo I).



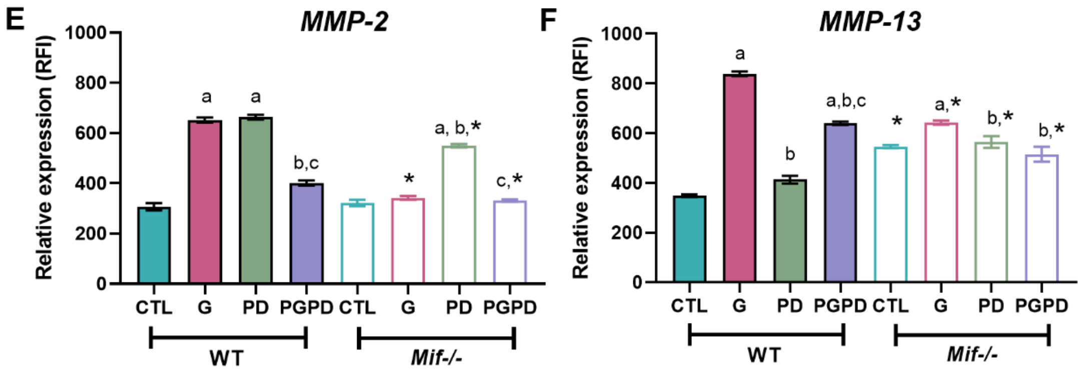


Fig. 6. Los ratones *Mif-/-* inducidos por periodontitis pregestacional muestran regulación negativa de la expresión de MMP-2 y MMP-13 durante la gestación. Inmunofluorescencia de MMP-2 (verde), MMP13 (rojo), Hotch (núcleos, Azul) presentes en el tejido periodontal, a nivel de la cresta ósea, indicada en el recuadro, para identificar: B (hueso), L (periodontal ligamento), G (encía), A. CTL WT y *Mif-/-*, B. PT WT y *Mif-/-*, C. P WT y *Mif-/-*, D, PGDP WT y *Mif-/-*, E) Expresión relativa de MMP-2, F) Expresión relativa de MMP-13. Se muestra una imagen representativa de tres experimentos independientes. Las imágenes fueron observadas a 63X. Se consideraron estadísticamente significativos valores de $p<0.05$, (a) comparados con ratones sanos (CTL), (b) comparados con grupo gestante (P), y (c) comparados con grupo periodontitis (PT), (*) *Mif-/-* vs WT, utilizando ANOVA con prueba de comparación múltiple de Tukey y prueba U de Mann-Whitney.

8.6 La periodontitis inducida en ratones Mif-/- expresan menores niveles de citocinas inflamatorias

En cuanto a la cuantificación de los transcritos *in situ* por qPCR, los datos nos permitieron confirmar que la periodontitis induce la sobreexpresión de MIF, así como que la gestación favorece la sobreexpresión de MIF como lo reportan Ietta y Paulesu (72, 77), (Fig 2 Anexo I), en los grupos WT la expresión de *tnf-α* fue significativamente mayor en todos los grupos, para *inf-γ* la expresión fue menor en todos los grupos con respecto al control, mientras que la ausencia de MIF disminuye los trascritos de *tnf-α* e *ifn-γ* y la sobreexpresión de *il-17*, probablemente debido a la remodelación presentada en los grupos con PT (73) (Fig 7 Anexo I).

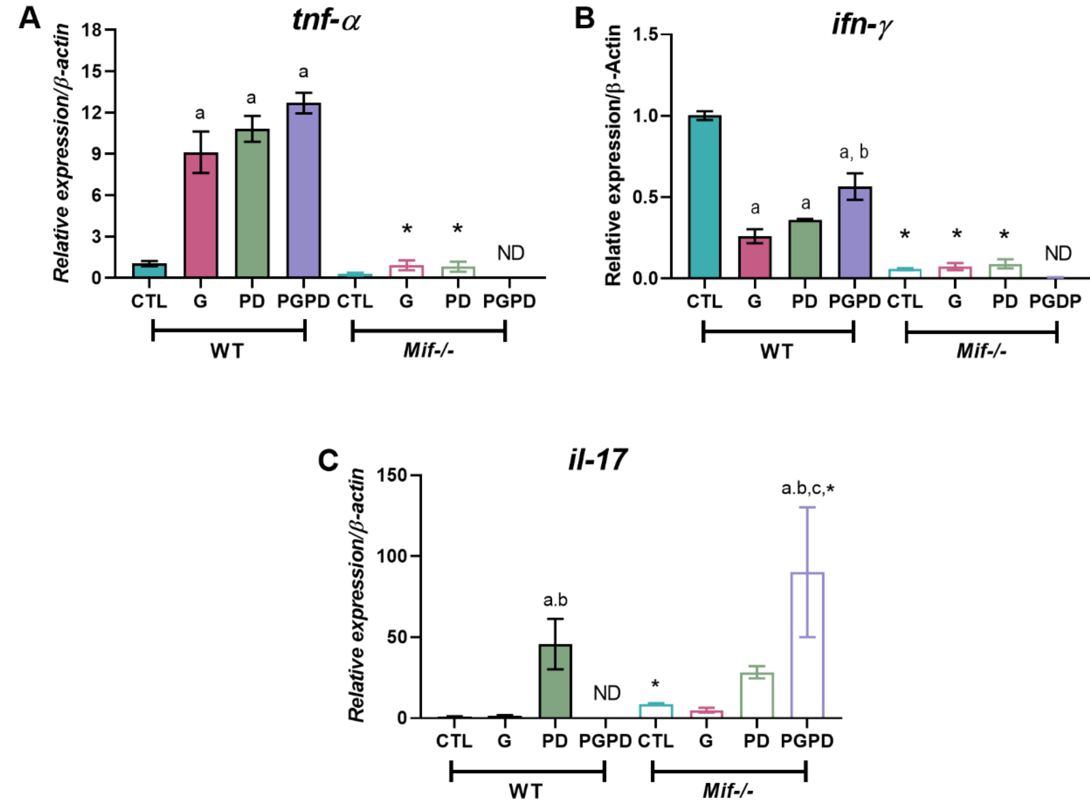


Fig. 7. La eliminación de MIF disminuye la expresión de *tnf-α* e *ifn-γ* pero no la expresión de *il-17* en la periodontitis. Se sacrificaron ratones hembra con periodontitis (PT) y ratones hembra con periodontitis pregestacional (PGPD) 9 semanas después de la inducción de periodontitis; la expresión de MIF en tejido periodontal se determinó mediante RT-qPCR. Se utilizaron como controles ratones sanos (CTL) y gestantes (P). Los datos se expresan como media ± SEM y son representativos de dos experimentos independientes con al menos tres ratones por grupo. Se consideraron estadísticamente significativos valores de $p<0.05$, (a) comparados con ratones sanos (CTL), (b) comparados con grupo gestante (P), y (c) comparados con grupo periodontitis (PT), (*) *Mif-/-* vs WT, (ND) No detectado, utilizando ANOVA con la prueba de comparación múltiple de Tukey y la prueba U de Mann-Whitney.

8.7 La periodontitis pregestacional en ratones Mif-/. presenta menor actividad de MMP-13

Siguientes resultados no fueron publicados, sin embargo, nos dan una idea de la actividad de MMP-13 disminuida principalmente en el grupo PGPD WT (Fig 8 A, B) en su forma pro-MMP-13, y sobreactivada en los grupos gestantes G WT y PGPD WT (Fig. 8 A, C), mientras que en los grupos *Mif-/-* hay una disminución de la actividad de esta enzima (Fig 8, A, C).

Estos resultados coinciden con los datos de la expresión de MMP-13 por inmunofluorescencia.

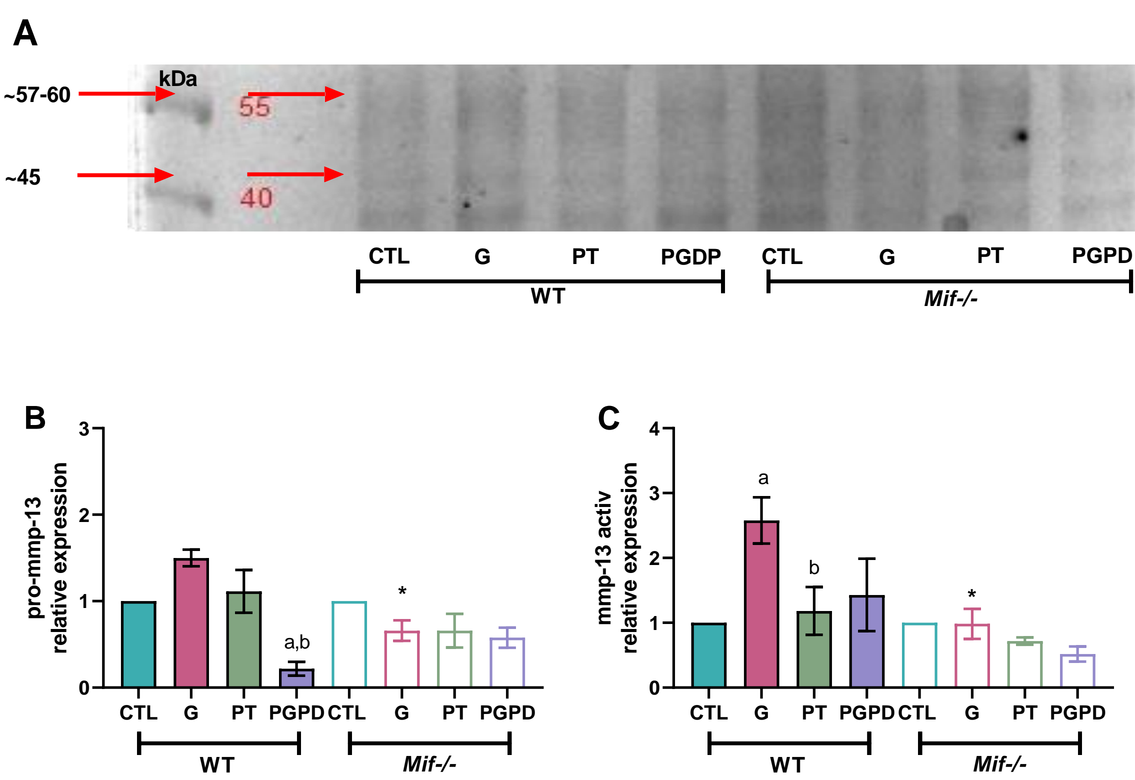


Fig. 8. La periodontitis pregestacional presenta menor actividad de MMP-13 en ratones *Mif-/-*. Ratones hembra con periodontitis (PT) y ratones hembra con periodontitis pregestacional (PGPD) fueron eutanizados a las 9 semanas posinducción de periodontitis, ratones hembra sanos (CTL) y, gestantes (G), fueron utilizados como control; zimograma para, **A)** gel en gel de poliacrilamida con sustrato de colágena, **B)** pro-MMp-13 (~57-60 kDa) **C)** MMP-13 activa (~56-45 kDa), grupos CTL WT, PT WT, P WT y PGPD WT, CTL *Mif-/-*, PT *Mif-/*, G *Mif-/-* y PGPD *Mif-/-*. Imagen representativa dos experimentos independientes. Valores de $p<0.05$ fueron considerados significativos, (a) comparados con los ratones sanos (CTL), (b) comparado con el grupo gestante (G), (c) comparado con el grupo con periodontitis (PT), (*) *Mif-/-* vs WT, utilizando ANOVA con una prueba de comparaciones múltiples de Tukey y la prueba U de Mann–Whitney.

8.8 La periodontitis pregestacional ratones *Mif-/-* presenta mayor actividad de MMP-2.

Por otra parte, la actividad de la MMP-2 para los grupos WT se vio ligeramente disminuida, mientras que se encuentra sobreactivada en los grupos *Mif-/-* (Fig 9, A, B, C), lo cual justifica la reconfiguración de las fibras de colágeno, principalmente el en grupo PGPD *Mif-/-*. Cabe

recordar, que la MMP-13 se activa primero para degradar colágena tipo I, II, III, para convertirlo en el sustrato que degrada MMP-2, principalmente fibronectina y elastina, sin embrago falta profundizar en estas observaciones.

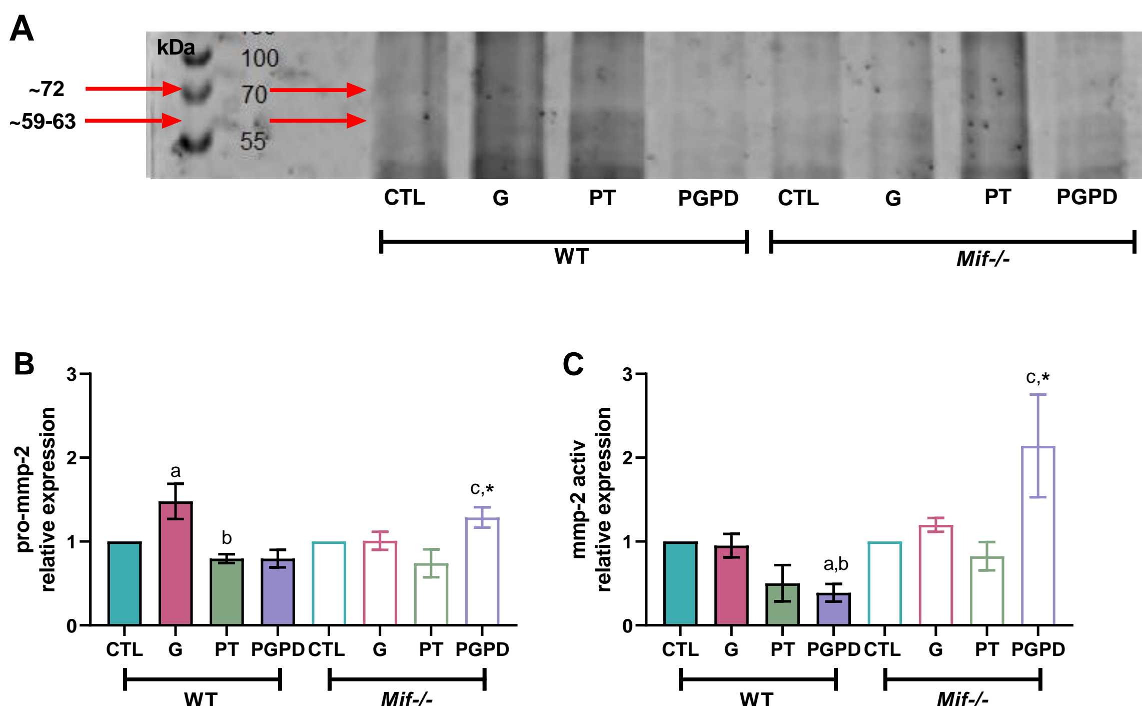
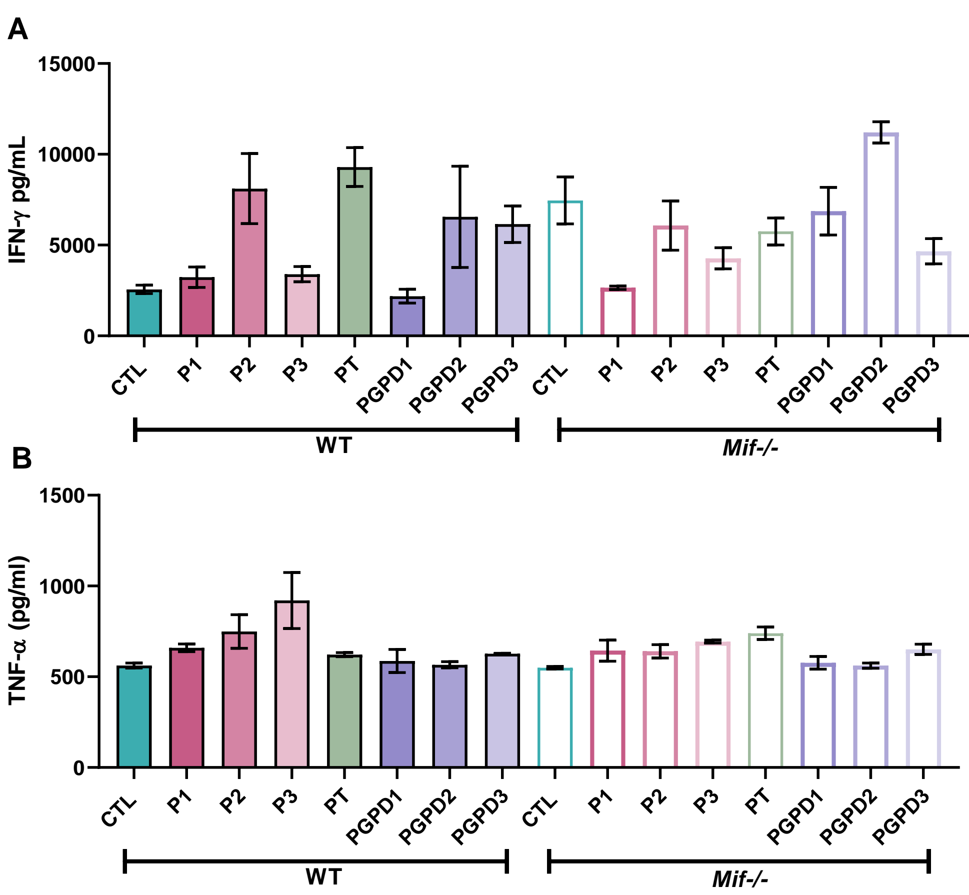


Fig. 9. La periodontitis pregestacional presenta mayor activación de MMP-2 en ratones *Mif-/-*. Ratones hembra con periodontitis (PT) y ratones hembra con periodontitis pregestacional (PGPD) fueron eutanizados a las 9 semanas posinducción de periodontitis, ratones hembra sanos (CTL) y, gestantes (G), fueron utilizados como control; zimograma para, **A)** gel en gel de poliacrilamida con sustrato de colágena, **B)** MMP-2 pro-enzima (~72 kDa), **C)** MMP-2 activa (~59-63 kDa). Grupos CTL WT, PT WT, P WT y PGPD WT, CTL *Mif-/-*, PT *Mif-/*, G *Mif-/-* y PGDP *Mif-/-*. Imagen representativa dos experimentos independientes. Valores de $p<0.05$ fueron considerados significativos, (a) comparados con los ratones sanos (CTL), (b) comparado con el grupo gestante (G), (c) comparado con el grupo con periodontitis (PT), (*) *Mif-/-* vs WT, utilizando ANOVA con la prueba de comparaciones múltiples de Tukey y la prueba U de Mann–Whitney.

8.9 La periodontitis no aumenta la concentración de IFN-γ, TNF-α, IL-6 e IL-10 en suero

Adicionalmente, para explorar la participación de MIF, cuantificamos la concentración de citocinas en suero, TNF-α, IFN-γ, IL-6 e IL-10, con sueros obtenidos de los ratones gestantes,

en los grupos G y PGPD en diferentes momentos: antes de la gestación (1), durante la gestación (2) y al final de la gestación (3), y para los grupos CTL y PT al momento de la eutanización. Aunque no encontramos diferencias en la concentración entre los diferentes grupos, si observamos algunas tendencias, como el aumento en la concentración de IFN-γ en los grupos PT WT, G WT en G2, y el grupo PGPD WT en PGPD G2 y PGPD G3, lo cual refiere una sobreproducción que se mantiene a lo largo de la gestación y continúa hasta el final de la gestación. En los grupos Mif-/- únicamente se observa aumento en la concentración en PGPD G2 en IFN-γ, lo cual parece estar relacionado con la gestación, más que con el desarrollo de la periodontitis o que sean *Mif-/-* (Fig 10 A). No identificamos variaciones en la concentración de TNF-α, IL-6 e IL-10 (Fig 10 B, C, D). Lo cual parece indicar que el modelo no provoca cambios sistémicos relevantes.



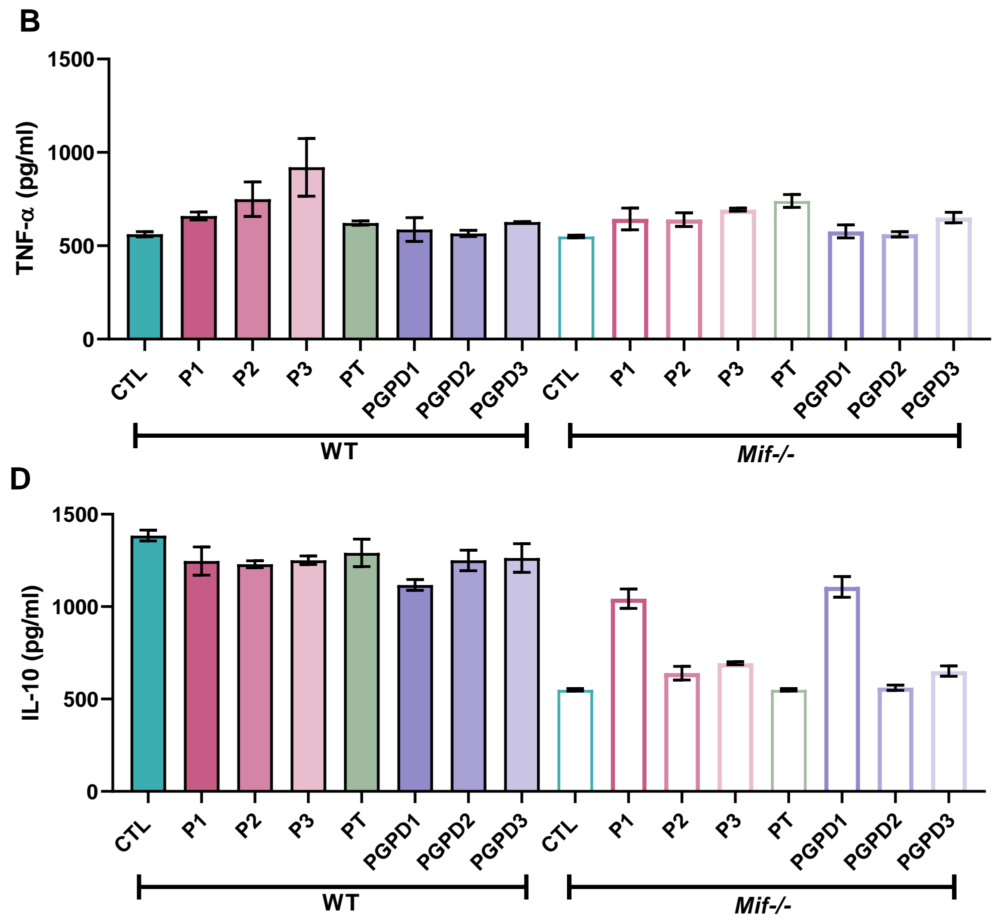


Fig. 10. La periodontitis no aumenta la concentración de IFN-γ, TNF-α, IL-6 e IL-10 en suero. Las concentraciones de citocinas fueron determinadas por ELISA en suero, en ratones hembra, sanos (CTL) y gestantes (G) como controles, ratones hembra con periodontitis (PT), y con periodontitis pregestacional (PGPD) los sueros se recolectaron por semana antes de la gestación (1), durante la gestación (2) y al final de la gestación (3). Los datos son expresados como la media $\pm$ SEM y son representativos de dos experimentos independientes con tres ratones por grupo. Valores de $p < 0.05$ fueron considerados como estadísticamente significativos (a) comparados con grupo sano (CTL), (b) comparado con el grupo gestante (G), y (c) comparado con el grupo con periodontitis (PT), utilizando una ANOVA con la prueba de Tukey de comparaciones múltiples. *Mif-/-* vs WT con la prueba U de Mann-Whitney.

9 Discusión

Se ha reportado la sobreexpresión de MIF en periodontitis y durante la gestación (54, 78). Sin embrago el papel de MIF durante el desarrollo y/o el agravamiento de la enfermedad periodontal durante la gestación no había sido descrito, nosotros desarrollamos un modelo de periodontitis experimental para estudiar la participación de MIF en el desarrollo de la enfermedad periodontal previo y durante la gestación en un modelo murino.

Nuestros resultados mostraron que el comportamiento de la enfermedad periodontal en nuestro modelo murino es diferente entre los diferentes grupos.

Inicialmente identificamos el aumento de los transcritos de *Mif* en tejidos periodontales con enfermedad periodontal, nuestros resultados coinciden a lo reportado por Zang et al. (71), estos niveles aumentaron al doble durante la gestación como reportaron Ietta y Paulesu (72, 77), y éste aumento persistió hasta 4 semanas después del nacimiento de las crías (Fig. 2 Anexo I).

El análisis de los tejidos periodontales nos permitió identificar el desarrollo de la enfermedad periodontal entre los diferentes grupos experimentales. De manera interesante, los ratones con periodontitis PT y PGPD presentaron mayor severidad en los parámetros clínicos e histológicos que cuantificamos. Mientras que los grupos *Mif-/-* no presentaron daño importante, o perdida de tejido, pero presentaron remodelación de los tejidos periodontales (Fig. 3 y 4, Anexo I).

El análisis histométrico nos permitió corroborar que la presencia de MIF contribuye a la exacerbación de la PT en la gestación, es importante aclarar que la perdida de tejido fue más amplia en la cara palatina que en la cara vestibular, probablemente debido a que la cara vestibular en ratones es más amplia (Fig 4A, B, C. Anexo I).

Aunque estos resultados no coindicen con Hays y cols., quienes reportaron mayor severidad en el grupo periodontitis y gestación con respecto al grupo solo periodontitis, sin embargo nuestro modelo presenta algunas diferencias, por ejemplo la inducción se realizó 8 días después de la confirmación de la gestación (79), mientras que nosotros

realizamos la inducción dos semas antes de la cruza, y se permitió la evolución a la lactancia, lo que puede favorecer la recuperación de los tejidos periodontales, fenómeno que González-Jaranay ha reportado en humanos (24).

La remodelación de los tejidos periodontales, principalmente en los grupos PT *Mif-/-* y PGPD *Mif-/-*, se caracterizó por la redistribución de las fibras de colágeno, el engrosamiento de las fibras de Sharpey, esto podría estar relacionado con una etapa de fibrosis, relacionado posiblemente con la activación excesiva de los fibroblastos, que podrían estar produciendo matriz extracelular. Estas observaciones deben ser profundizadas (Fig 5, Anexo I).

La remodelación de la matriz extracelular está relacionada con las MMPs, nosotros medimos la presencia y actividad del MMP-13 y MMP-2, que son dos de las enzimas que participan en la degradación y remodelación de la matriz extracelular. Nuestros resultados mostraron que hay una disminución en la expresión y actividad de MMP-2 en los grupos PT *Mif-/-* y P *Mif-/-*, así como una sobreexpresión en los grupos P WT y PT WT. De manera interesante, el grupo PGPD WT no presentó cambios en la expresión de MMP-2 con respecto al control, lo que podría estar relacionado a la inmunoregulación que provoca la gestación (Fig. 6, Anexo I, Fig 4). Mientras que para MMP-13, la expresión fue mayor en los grupos P WT y PGPD WT con respecto a los grupos CTL WT y PT WT, sin embargo, para los grupos *Mif -/-* la expresión basal fue mayor, y aunque no presentaron cambios en la expresión en los diferentes grupos (Fig. 6, Anexo I, Fig 4).

Esto puede relacionarse con la presencia de citocinas, aunque no encontramos variaciones en las concentraciones sistémicas de TNF-α, INF-γ, IL-6 e IL-10, si encontramos sobreexpresión *mif, tnf-α* e *il-17 in situ* en el grupo PT WT, consistente con las características clínicas e histológicas que coinciden con lo reportado por Mesa y cols. en pacientes con PT (80). Mientras que la ausencia de MIF, disminuyó la expresión de *tnf-α* e *Ifn-γ* en el grupo PT *Mif -/-* y la sobreexpresión de *il-17*, esta última con menor intensidad que en el grupo PT WT, debido tal vez a la remodelación tisular, que se asocia a la integridad epitelial donde participa IL-17(81) .

La respuesta inmune depende su interacción con el microbioma, a través de los PRRs, MIF favorece la expresión de TLR-4 (51), el cual reconoce al LPS de bacterias gram negativas, el microbioma periodontopatógeno es principalmente de este tipo, por lo que establecer la configuración del microbioma en este modelo ayudaría a comprender el papel de MIF en el desarrollo de la PT.

Un factor que también interviene, tanto en la composición del microbioma, como en la modulación del sistema inmune son las variaciones hormonales que suceden durante la gestación. Por lo que correlacionar los perfiles hormonales y la expresión de citocinas en diferentes momentos de la gestación podría ayudar a comprender los efectos de la gestación sobre el desarrollo de la EP.

10 Conclusión

Nuestro estudio sugiere que MIF exacerba la periodontitis experimental previa y durante la gestación en un modelo murino, principalmente cuando esta se encuentra presente previo a la gestación, al favorecer la sobreexpresión de TNF-α y MMP-13. La ausencia de MIF demostró que esta citocina tiene una fuerte influencia en el agravamiento de la enfermedad periodontal preexistente durante el embarazo, ya que los ratones gestantes *Mif-/-* desarrollaron una enfermedad menos grave que los ratones gestantes WT. Además, también demostramos que MIF regula la expresión de TNF-α y la actividad de MMP-13. Lo que sugiere que MIF es responsable de forma parcial de la inflamación asociada a la severidad de la periodontitis durante la gestación.

11 Referencias Bibliográficas

1. Enwonwu CO, Salako N. The periodontal disease-systemic health-infectious disease axis in developing countries. Periodontol 2000. 2012;60(1):64-77.
2. Papapanou PN, Sanz M, Buduneli N, Dietrich T, Feres M, Fine DH, et al. Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. J Periodontol. 2018;89 Suppl 1:S173-S82.
3. Slavkin HC. Does the mouth put the heart at risk? J Am Dent Assoc. 1999;130(1):109-13.
4. Tonetti MS, Jepsen S, Jin L, Otomo-Corgel J. Impact of the global burden of periodontal diseases on health, nutrition and wellbeing of mankind: A call for global action. Journal of clinical periodontology. 2017;44(5):456-62.
5. Nazir MA. Prevalence of periodontal disease, its association with systemic diseases and prevention. International journal of health sciences. 2017;11(2):72.
6. Duque A. Prevalencia de periodontitis crónica en Iberoamérica. Revista clínica de periodoncia, implantología y rehabilitación oral. 2016;9(2):208-15.
7. Li Y, Lee S, Hujoel P, Su M, Zhang W, Kim J, et al. Prevalence and severity of gingivitis in American adults. American journal of dentistry. 2010;23(1):9-13.
8. Murillo G, Vargas MA, Castillo J, Serrano JJ, Ramirez GM, Viales JH, et al. Prevalence and Severity of Plaque-Induced Gingivitis in Three Latin American Cities: Mexico City-Mexico, Great Metropolitan Area-Costa Rica and Bogota-Colombia. 2018;20(2):91-102.
9. The pathogenesis of periodontal diseases. J Periodontol. 1999;70(4):457-70.
10. Liu YC, Lerner UH, Teng YT. Cytokine responses against periodontal infection: protective and destructive roles. Periodontol 2000. 2010;52(1):163-206.
11. Genco RJ. Current view of risk factors for periodontal diseases. J Periodontol. 1996;67(10 Suppl):1041-9.
12. Webb DA, Mathew L, Culhane JF. Lessons learned from the Philadelphia Collaborative Preterm Prevention Project: the prevalence of risk factors and program participation rates among women in the intervention group. BMC Pregnancy Childbirth. 2014;14:368.
13. Balan P, Chong YS, Umashankar S, Swarup S, Loke WM, Lopez V, et al. Keystone Species in Pregnancy Gingivitis: A Snapshot of Oral Microbiome During Pregnancy and Postpartum Period. Front Microbiol. 2018;9:2360.
14. Ortiz-Sánchez BJ, Legorreta-Herrera M, Rodriguez-Sosa M. Influence of Gestational Hormones on the Bacteria-Induced Cytokine Response in Periodontitis. Mediators of Inflammation. 2021;2021:5834608.
15. Kriebel K, Hieke C, Muller-Hilke B, Nakata M, Kreikemeyer B. Oral Biofilms from Symbiotic to Pathogenic Interactions and Associated Disease -Connection of Periodontitis and Rheumatic Arthritis by Peptidylarginine Deiminase. Front Microbiol. 2018;9:53.
16. Do T, Devine D, Marsh PD. Oral biofilms: molecular analysis, challenges, and future prospects in dental diagnostics. Clin Cosmet Investig Dent. 2013;5:11-9.
17. Radaic A, Kapila YL. The oralome and its dysbiosis: New insights into oral microbiome-host interactions. Comput Struct Biotechnol J. 2021;19:1335-60.
18. Kornman KS, Loesche WJ. Effects of estradiol and progesterone on Bacteroides melaninogenicus and Bacteroides gingivalis. Infect Immun. 1982;35(1):256-63.
19. Wu M, Chen SW, Su WL, Zhu HY, Ouyang SY, Cao YT, et al. Sex Hormones Enhance Gingival Inflammation without Affecting IL-1beta and TNF-alpha in Periodontally Healthy Women during Pregnancy. Mediators Inflamm. 2016;2016:4897890.

20. Silva de Araujo Figueiredo C, Gonçalves Carvalho Rosalem C, Costa Cantanhede AL, Abreu Fonseca Thomaz ÉB, Fontoura Nogueira da Cruz MC. Systemic alterations and their oral manifestations in pregnant women. Journal of Obstetrics and Gynaecology Research. 2017;43(1):16-22.
21. Fusco NdS, Foratori-Junior GA, Missio ALT, Jesuino BG, Sales-Peres SHdC. Systemic and oral conditions of pregnant women with excessive weight assisted in a private health system. International dental journal. 2019;69(6):472-9.
22. Duan X, Hays A, Zhou W, Sileewa N, Upadhyayula S, Wang H, et al. Porphyromonas gingivalis induces exacerbated periodontal disease during pregnancy. Microbial pathogenesis. 2018;124:145-51.
23. Hays A, Duan X, Zhu J, Zhou W, Upadhyayula S, Shivde J, et al. Down-regulated Treg cells in exacerbated periodontal disease during pregnancy. International immunopharmacology. 2019;69:299-306.
24. González-Jaranay M, Téllez L, Roa-López A, Gómez-Moreno G, Moreu G. Periodontal status during pregnancy and postpartum. PLoS One. 2017;12(5):e0178234.
25. Martínez-Martínez RE, Moreno-Castillo DF, Loyola-Rodríguez JP, Sánchez-Medrano AG, San Miguel-Hernández JH, Olvera-Delgado JH, et al. Association between periodontitis, periodontopathogens and preterm birth: is it real? Archives of gynecology and obstetrics. 2016;294(1):47-54.
26. Fogacci MF, de OC Cardoso E, Barbirato DdS, de Carvalho DP, Sansone C. No association between periodontitis and preterm low birth weight: a case–control study. Archives of gynecology and obstetrics. 2018;297(1):71-6.
27. Wimmer G, Pihlstrom BL. A critical assessment of adverse pregnancy outcome and periodontal disease. Journal of clinical periodontology. 2008;35:380-97.
28. Seraphim APCG, Chiba FY, Pereira RF, Mattera MSdLC, Moimaz SAS, Sumida DH. Relationship among periodontal disease, insulin resistance, salivary cortisol, and stress levels during pregnancy. Brazilian dental journal. 2016;27(2):123-7.
29. Daalderop L, Wieland B, Tomsin K, Reyes L, Kramer B, Vanterpool S, et al. Periodontal disease and pregnancy outcomes: overview of systematic reviews. JDR Clinical & Translational Research. 2018;3(1):10-27.
30. Hegde R, Awan K. Effects of periodontal disease on systemic health. Disease-a-Month. 2018.
31. Pérez-Molina JJ, González-Cruz MJ, Panduro-Barón JG, Santibáñez-Escobar LP, Quezada-Figueroa NA, Bedolla-Barajas M. Enfermedad periodontal como factor de riesgo adicional asociado con nacimiento pretérmino en México: un estudio de casos y controles. Gaceta medica de Mexico. 2019;155(2):143-8.
32. Foratori-Junior GA, da Silva BM, da Silva Pinto AC, Honório HM, Groppo FC, de Carvalho Sales-Peres SH. Systemic and periodontal conditions of overweight/obese patients during pregnancy and after delivery: a prospective cohort. Clinical oral investigations. 2020;24(1):157-65.
33. Teshome A, Yitayeh A. Relationship between periodontal disease and preterm low birth weight: systematic review. Pan African Medical Journal. 2016;24(1).
34. Song B, Zhang Y, Chen L, Zhou T, Huang W, Zhou X, et al. The role of Toll-like receptors in periodontitis. 2017;23(2):168-80.
35. Di Benedetto A, Gigante I, Colucci S, Grano MJC, Immunology D. Periodontal disease: linking the primary inflammation to bone loss. 2013;2013.
36. Mahanonda R, Pichyangkul SJP. Toll-like receptors and their role in periodontal health and disease. 2007;43(1):41-55.

37. Matsuki Y, Yamamoto T, Hara K. Detection of inflammatory cytokine messenger RNA (mRNA)-expressing cells in human inflamed gingiva by combined in situ hybridization and immunohistochemistry. Immunology. 1992;76(1):42-7.
38. Yamazaki K, Nakajima T, Gemmell E, Polak B, Seymour GJ, Hara K. IL-4- and IL-6-producing cells in human periodontal disease tissue. J Oral Pathol Med. 1994;23(8):347-53.
39. Diabetes and periodontal diseases. Committee on Research, Science and Therapy. American Academy of Periodontology. J Periodontol. 2000;71(4):664-78.
40. Beklen A, Ainola M, Hukkanen M, Gürgan C, Sorsa T, Konttinen YT. MMPs, IL-1, and TNF are regulated by IL-17 in periodontitis. Journal of dental research. 2007;86(4):347-51.
41. Mahanonda R, Jitprasertwong P, Sa-Ard-Iam N, Rerkyen P, Charatkulangkun O, Jansisyanont P, et al. Effects of IL-17 on human gingival fibroblasts. Journal of dental research. 2008;87(3):267-72.
42. Parachuru V, Coates D, Milne T, Rich A, Seymour G. FoxP3+ regulatory T cells, interleukin 17 and mast cells in chronic inflammatory periodontal disease. Journal of periodontal research. 2018;53(4):622-35.
43. Cardoso C, Garlet G, Crippa G, Rosa A, Junior W, Rossi M, et al. Evidence of the presence of T helper type 17 cells in chronic lesions of human periodontal disease. Oral microbiology and immunology. 2009;24(1):1-6.
44. Kini V, Mohanty I, Telang G, Vyas N. Immunopathogenesis and distinct role of Th17 in periodontitis: A review. Journal of oral biosciences. 2022;64(2):193-201.
45. Rose L, Genco R, Cohen W. Periodontal Medicine. Periodontal Medicine and the female patient. BC Decker Inc Canada. 2000:162-5.
46. Jensen J, Liljemark W, Bloomquist C. The effect of female sex hormones on subgingival plaque. J Periodontol. 1981;52(10):599-602.
47. Mealey BL, Moritz AJ. Hormonal influences: effects of diabetes mellitus and endogenous female sex steroid hormones on the periodontium. Periodontol 2000. 2003;32:59-81.
48. Kudrin A, Ray D. Cunning factor: macrophage migration inhibitory factor as a redox-regulated target. Immunology and cell biology. 2008;86(3):232-8.
49. Flaster H, Bernhagen Jr, Calandra T, Bucala R. The macrophage migration inhibitory factor-glucocorticoid dyad: regulation of inflammation and immunity. Molecular Endocrinology. 2007;21(6):1267-80.
50. Grieb G, Merk M, Bernhagen J, Bucala R. Macrophage migration inhibitory factor (MIF): a promising biomarker. Drug news & perspectives. 2010;23(4):257.
51. Roger T, David J, Glauser MP, Calandra T. MIF regulates innate immune responses through modulation of Toll-like receptor 4. Nature. 2001;414(6866):920-4.
52. Offenbacher S, Lieff S, Boggess KA, Murtha A, Madianos P, Champagne C, et al. Maternal periodontitis and prematurity. Part I: Obstetric outcome of prematurity and growth restriction. Annals of periodontology. 2001;6(1):164-74.
53. Marsh LM, Cakarova L, Kwapiszewska G, von Wulffen W, Herold S, Seeger W, et al. Surface expression of CD74 by type II alveolar epithelial cells: a potential mechanism for macrophage migration inhibitory factor-induced epithelial repair. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2009;296(3):L442-L52.
54. Ietta F, Todros T, Ticconi C, Piccoli E, Zicari A, Piccione E, et al. Macrophage migration inhibitory factor in human pregnancy and labor. American Journal of Reproductive Immunology. 2002;48(6):404-9.
55. Morimoto T, Nishihira J, Kohgo T. Immunohistochemical localization of macrophage migration inhibitory factor (MIF) in human gingival tissue and its pathophysiological functions. Histochemistry and cell biology. 2003;120(4):293-8.

56. Ortiz-Garcia YM, Garcia-Iglesias T, Morales-Velazquez G, Lazalde-Ramos BP, Zuniga-Gonzalez GM, Ortiz-Garcia RG, et al. Macrophage Migration Inhibitory Factor Levels in Gingival Crevicular Fluid, Saliva, and Serum of Chronic Periodontitis Patients. Biomed Res Int. 2019;2019:7850392.
57. Zhang D, Xu T, Xu Q, Dong Q, Luo Y, Gao L, et al. Expression profile of macrophage migration inhibitory factor in periodontitis. 2021;122:105003.
58. Yilmaz D, Gönüllü E, Gürsoy M, Könönen E, Gürsoy UKJJoP. Salivary and serum concentrations of monocyte chemoattractant Protein-1, macrophage inhibitory factor and fractalkine in relation to rheumatoid arthritis and periodontitis. 2020.
59. Li X, Lan H, Huang X, Zhang C, Jin L. Expression profile of macrophage migration-inhibitory factor in human gingiva and reconstituted human gingival epithelia stimulated by Porphyromonas gingivalis lipopolysaccharide. Journal of periodontal research. 2013;48(4):527-32.
60. Madeira MFM, Queiroz-Junior CM, Costa GM, Santos PC, Silveira EM, Garlet GP, et al. MIF induces osteoclast differentiation and contributes to progression of periodontal disease in mice. Microbes and infection. 2012;14(2):198-206.
61. Hirschfeld J, Howait M, Movila A, Parčina M, Bekeredjian-Ding I, Deschner J, et al. Assessment of the involvement of the macrophage migration inhibitory factor–glucocorticoid regulatory dyad in the expression of matrix metalloproteinase-2 during periodontitis. European Journal of Oral Sciences. 2017;125(5):345-54.
62. Bamashmous S, Kotsakis GA, Jain S, Chang AM, McLean JS, Darveau RP. Clinically Healthy Human Gingival Tissues Show Significant Inter-individual Variability in GCF Chemokine Expression and Subgingival Plaque Microbial Composition. Front Oral Health. 2021;2:689475.
63. Cascales Angosto M, Angel Alvarez-Gomez J, editors. Metalloproteinases, extracellular matrix and cancer. ANALES DE LA REAL ACADEMIA NACIONAL DE FARMACIA; 2010: REAL ACAD NACIONAL FARMACIA FARMACIA, 9 Y 11, MADRID, SPAIN.
64. Butler GS, Overall CM. Matrix metalloproteinase processing of signaling molecules to regulate inflammation. Periodontology 2000. 2013;63(1):123-48.
65. Kinane DF. Regulators of tissue destruction and homeostasis as diagnostic aids in periodontology. Periodontology 2000. 2000;24(1):215-25.
66. Sapna G, Gokul S, Bagri-Manjrekar K. Matrix metalloproteinases and periodontal diseases. Oral diseases. 2014;20(6):538-50.
67. Hernandez M, Valenzuela MA, Lopez-Otin C, Alvarez J, Lopez JM, Vernal R, et al. Matrix metalloproteinase-13 is highly expressed in destructive periodontal disease activity. Journal of periodontology. 2006;77(11):1863-70.
68. Inanc S, Keles D, Oktay G. An improved collagen zymography approach for evaluating the collagenases MMP-1, MMP-8, and MMP-13. Biotechniques. 2017;63(4):174-80.
69. Mäkelä M, Salo T, Uitto VJ, Larjava H. Matrix metalloproteinases (MMP-2 and MMP-9) of the oral cavity: cellular origin and relationship to periodontal status. J Dent Res. 1994;73(8):1397-406.
70. Coronato S, Laguens G, Di Girolamo V. Rol de las metaloproteinasas y sus inhibidores en patología tumoral. MEDICINA (Buenos Aires). 2012;72(6):495-502.
71. Zhang D, Xu T, Xu Q, Dong Q, Luo Y, Gao L, et al. Expression profile of macrophage migration inhibitory factor in periodontitis. Archives of oral biology. 2021;122:105003.
72. Paulesu L, Bhattacharjee J, Bechi N, Romagnoli R, Jantra S, Ietta F. Pro-inflammatory cytokines in animal and human gestation. Current pharmaceutical design. 2010;16(32):3601-15.
73. Ortiz-Sánchez BJ, Juárez-Avelar I, Andrade-Meza A, Mendoza-Rodríguez MG, Chirino YI, Monroy-Pérez E, et al. Periodontitis exacerbation during pregnancy in mice: Role of macrophage migration inhibitory factor as a key inductor. J Periodontal Res. 2023.

74. Benzen B, Grauballe MCB, Björnsson M, Stoltze K, Hjørting-Hansen E, Holmstrup P. A comparison of two models of experimental periodontitis in rats. Scandinavian Journal of Laboratory Animal Sciences. 2005;32(2):73-80.
75. Abe T, Hajishengallis G. Optimization of the ligature-induced periodontitis model in mice. J Immunol Methods. 2013;394(1-2):49-54.
76. Semenoff TA, Semenoff-Segundo A, Bosco AF, Nagata MJ, Garcia VG, Biasoli ER. Histometric analysis of ligature-induced periodontitis in rats: a comparison of histological section planes. J Appl Oral Sci. 2008;16(4):251-6.
77. Ietta F, Bechi N, Romagnoli R, Bhattacharjee J, Realacci M, Di Vito M, et al. 17β-Estradiol modulates the macrophage migration inhibitory factor secretory pathway by regulating ABCA1 expression in human first-trimester placenta. American Journal of Physiology-Endocrinology and Metabolism. 2010;298(3):E411-E8.
78. Gürkan A, Eren G, Çetinkalp Ş, Akçay YD, Emingil G, Atilla G. Monocyte chemotactic protein-1, RANTES and macrophage migration inhibitory factor levels in gingival crevicular fluid of metabolic syndrome patients with gingivitis. Archives of oral biology. 2016;69:82-8.
79. Hays A, Duan X, Zhu J, Zhou W, Upadhyayula S, Shivde J, et al. Down-regulated Treg cells in exacerbated periodontal disease during pregnancy. International immunopharmacology. 2019;69:299-306.
80. Mesa F, Pozo E, O'Valle F, Puertas A, Magan-Fernandez A, Rosel E, et al. Relationship between periodontal parameters and plasma cytokine profiles in pregnant woman with preterm birth or low birth weight. Clinical oral investigations. 2016;20(4):669-74.
81. Abusleme L, Moutsopoulos NM. IL-17: overview and role in oral immunity and microbiome. Oral Dis. 2017;23(7):854-65.

12 Anexo I Articulo Requisito

Received: 12 June 2023 | Revised: 12 October 2023 | Accepted: 30 October 2023
DOI: 10.1111/jre.13211

ORIGINAL ARTICLE

WILEY

Periodontitis exacerbation during pregnancy in mice: Role of macrophage migration inhibitory factor as a key inductor

Betsaida J. Ortiz-Sánchez[1,2] | Imelda Juárez-Avelar[2,3] | Antonio Andrade-Meza[3,4] | Mónica Gabriela Mendoza-Rodríguez[5] | Yolanda I. Chirino[6] | Eric Monroy-Pérez[7] | Gloria Luz Paniagua-Contreras[7] | Miriam Rodriguez-Sosa[4]

[1]Carrera de Cirujano Dentista, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México (UNAM), Tlalnepantla, Mexico

[2]Programa de Doctorado en Ciencias Biológicas, Universidad Nacional Autónoma de México (UNAM), Ciudad de México, Mexico

[3]Laboratorio de Inmunidad Innata. Unidad de Investigación en Biomedicina, Facultad de Estudios Superiores Iztacala, UNAM, Tlalnepantla, Mexico

[4]Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM), Ciudad de México, Mexico

[5]Laboratorio de Inmunoparasitología. Unidad de Investigación en Biomedicina, Facultad de Estudios Superiores Iztacala, UNAM, Tlalnepantla, Mexico

[6]Laboratorio de Carcinogénesis y Toxicología. Unidad de Investigación en Biomedicina, Facultad de Estudios Superiores Iztacala, UNAM, Tlalnepantla, Mexico

[7]Facultad de Estudios Superiores Iztacala, UNAM, Tlalnepantla, Mexico

Correspondence
Miriam Rodriguez-Sosa, Unidad de Biomedicina, FES-Iztacala, UNAM. Av. De los Barrios 1, Los Reyes Iztacala, Tlalnepantla, Edo. de México, 54090, Mexico.
Email: rodriguezm@unam.mx

Funding information
Council for Science and Technology of the State of Mexico (COMECYT); Support Program for Research Projects and Technological Innovation (PAPIIT)-UNAM

Abstract

Objective: The present study was designed to investigate the role of macrophage migration inhibitory factor (MIF) in the exacerbation of pregestational periodontal disease (PGPD).

Background: Periodontitis (PT) is a severe stage of periodontal disease characterized by inflammation of the supporting tissues of the teeth, which usually worsens during pregnancy. MIF is a proinflammatory cytokine that is significantly elevated in periodontitis, both at the beginning and at the end of pregnancy. Although periodontitis usually presents with greater severity during pregnancy, the participation of MIF in the evolution of periodontitis has not been established.

Methods: To analyze the relevance of MIF in the exacerbation of PGPD, we employed a model of PGPD in WT and *Mif-/-* mice, both with a BALB/c genetic background. PT was induced with nylon suture ligatures placed supramarginally around the second upper right molar. For PGPD, PT was induced 2 weeks before mating. We evaluated histological changes and performed histometric analysis of the clinical attachment loss, relative expression of MMP-2 and MMP-13 by immunofluorescence, and relative expression of the cytokines *mif*, *tnf-α*, *ifn-γ*, and *il*-17 by quantitative real-time polymerase chain reaction (qRT-PCR).

Results: Our data revealed that periodontal tissue from PGPD WT mice produced a twofold increase in MIF compared with PT WT mice. Moreover, the evolution of periodontitis in *Mif-/-* mice was less severe than in PGDP WT mice. Periodontal tissue from *Mif-/-* mice with PGPD produced 80% less TNF-α and no IFN-γ, as well as 50% lower expression of matrix metalloproteinase (MMP)-2 and 25% less MMP-13 compared to WT PGDP mice.

Conclusions: Our study suggests that MIF plays an important role in the exacerbation of periodontitis during pregnancy and that MIF is partially responsible for the inflammation associated with the severity of periodontitis during pregnancy.

KEYWORDS
animal model, inflammatory mediator, innate immunology, matrix metalloproteinase, periodontal disease, periodontal immunology

© 2023 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd.

1 | INTRODUCTION

Periodontal disease is characterized by an inflammatory alteration of the supporting tissues of the teeth, such as the gum, alveolar bone, periodontal ligament (PDL), and root cementum.[1] It is a highly prevalent disease worldwide,[2] and approximately 60% of the population has some extent of periodontal disease.[3] In Latin America, the prevalence is reported to reach up to 90% in adults.[4] Approximately 38.4% of women have periodontal disease[5]; significantly, during pregnancy, women exhibit greater severity of the disease parameters, such as greater depth of probing and gingival bleeding.[6] It is well known that pregnancy results in significant changes in the immune response since the fetus must be tolerated.[7] Thus, pregnancy favors the emergence of several autoimmune and inflammatory diseases; likewise, periodontitis in pregnant women increases to 56%.[8]

Periodontal disease, in its more severe form, affects alveolar bone and is known as periodontitis (PT). The inflammatory process in PT is the driving force in the pathogenesis of the disease. The expression of inflammatory mediators such as TNF-α, IL-1β, IL-6, and IL-17,[9,10] along with matrix metalloproteinases (MMP)-1, MMP-3, MMP-8, MMP-13 (collagenases), and MMP-2 and MMP-9 (gelatinases), is involved in pathology and tissue remodeling, respectively.[11,12]

MIF is an inflammatory cytokine of the innate immune response,[13,14] and it is produced by various immune and non-immune cell types, such as monocytes, macrophages (Mφ), mast cells, eosinophils, epithelial cells, endothelium, and fibroblasts, among others.[15] MIF amplifies the immune response, stimulating the expression of mediators that are key in MIF-related inflammatory changes, such as TNF-α, IFN-γ, IL-1β, IL-2, IL-6, and IL-8[16]; therefore, MIF counteracts the anti-inflammatory action of glucocorticoids.[17] MIF is found preformed within cytoplasmic pools in some cells, such as adipocytes and Mφ; therefore, it does not require transcription activation and mRNA translocation for its immediate release after a first insult.[18] Once MIF is released, it induces the expression of TNF-α, IFN-γ, IL-17, MMP-2, and MMP-13.[16] MIF also enhances TLR-4 expression.[19,20] After this first response, MIF production and expression are induced in response to sustained antigenic challenges, mainly lipopolysaccharides (LPS), cytokines such as TNF-α and IFN-ɣ, and physiological stress.[21]

MIF expression has been detected in the epithelial tissue of patients with PT.[14,22] Moreover, high levels of MIF have been documented in the gingival crevicular fluid, saliva, and serum of individuals with periodontal disease.[23–25] The high levels of MIF, in samples from PT patients, suggest that it plays an important role in the physiopathology of periodontal tissues, mainly in free gingiva and junctional epithelium, as well as in keratinocytes, basal cells, and fibroblasts,[26] and probably also plays an important role in osteoclastogenesis.[27]

MIF is also expressed in reproductive tissues, and its expression responds to hormonal changes; for example, at low concentrations of estradiol, the expression of MIF is initiated, while at high concentrations of estradiol, the production of MIF is suppressed.[28] This explains why MIF overexpression has been associated with different stages of ovulation, the menstrual cycle, and pregnancy.[29,30]

Hormones present during pregnancy alter the physiological response of periodontal tissues; for example, gingival tissues are more sensitive, so the ranges of inflammation present fluctuate from slight edema and redness to severe inflammation with bleeding and gingival hyperplasia. In addition, exudate is favored in inflamed gingival tissues,[31] and little is known about its effects on the development of periodontitis. The interaction between the concentration of gestational hormones and periodontal disease seems to be bidirectional.[32]

Although pregnancy is an important factor in the exacerbation of periodontal disease, it has not yet been established whether the increase in MIF from pregnancy influences the aggravation of inflammatory pathology associated with PT. Here, we studied the influence of the expression of gestational MIF on the aggravation of periodontitis existing before pregnancy.

2 | EXPERIMENTAL DESIGN

2.1 | Animals

Six- to eight-week-old female *Mif-/-* mice on a BALB/c genetic background were backcrossed for more than 10 generations.[33] Age-matched WT BALB/c female mice were purchased from Invigo (Invigo, Mexico City, Mexico). Mice were maintained in a pathogen-free environment at the FES-Iztacala, UNAM animal facilities. Genotyping of *Mif-/-* mice was routinely performed on genomic DNA isolated from tail samples using a polymerase chain reaction (PCR) procedure.[34] PCR was performed using the following primers: MIF: forward 5′-AGACCACGTGCTTAGCTGAG-3′, reverse 5′-GCATCGCTACCGGTGGATAA-3′, neomycin (NEO): forward 5′-ATTGAACAAGATGGATTGCAC-3′, reverse 5′-ATACTTTCTCGGCAGGAGCA-3′ (all synthesized by Sigma-Aldrich, Mexico City, Mexico). PCR for the amplification of MIF and NEO was performed with Taq DNA polymerase (Ampliqon, Bioreagents and Molecular Diagnostics, Denmark) following the manufacturer's instructions. A PCR fragment of 200 bp, corresponding to MIF, or 500 bp, corresponding to NEO, was visualized to identify WT or *Mif-/-* mice, respectively. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel and viewed under UV light (Bio-Rad, Hercules, CA, USA). The genotype confirmation electrophoresis image is available in Figure S1.

These studies were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals adopted by the U.S. National Institutes of Health, and the Mexican Regulation of Animal Care and maintenance (NOM-062-ZOO-1999, 2001). Our protocol was approved by the Ethics Committee at FES-Iztacala, UNAM CE/FESI/042022/1465.

2.2 | Periodontitis induction

PT was induced following a previously reported protocol.[35,36] Briefly, nylon 6-0 suture ligatures (Atramat, Internacional Farmacéutica, Mexico City, Mexico) were placed supramarginally around the second upper right molar with a surgical microscope (Carl Zeiss GmbH Berlin,

Germany) in experimental mice and under deep anesthesia with xylazine (Porcine, Pisa, Jalisco, Mexico) at 10 mg/kg I.M. and ketamine (Anesket, Pisa) at 2 mg/kg I.M. The permanence of the nylon ligatures was verified every week. Representative image is available in Figure S2.

2.3 | Experimental groups

Mice were grouped as follows: (1) control group (CTL) consisted of age-matched mice without PT and non-gestational mice, (2) group of gestational mice (P) (3 weeks of gestation +4 weeks of weaning of the pups), (3) mice induced PT but non-gestational (PT), and (4) mice induced PT 2 weeks before breeding (PGPD), pregnancy was verified by the presence of vaginal plug (induction of periodontitis 2 weeks + gestation 3 weeks + weaning of pups, 4 weeks). After 9 weeks of periodontitis induction, 4 weeks after offspring were weaned, and the mice were euthanized in a CO_2 chamber (three to five mice per group, two independent experiments; Figure 1).

2.4 | Histology

Hematoxylin and eosin (H&E) staining was performed on coronal sections of the second right molar to observe periodontal tissues and measure clinical attachment loss (CAL), and the results were compared between experimental groups. Briefly, maxillae were recovered and fixed in 4% paraformaldehyde solution (Sigma-Aldrich), decalcified in 4% EDTA solution (Sigma-Aldrich) for 20 days, and then embedded in paraffin. Sequential 5-µm sections were made in the second molar area and stained with H&E (Sigma-Aldrich) and Masson's trichrome (Sigma-Aldrich).

The stained histological sections were observed under an optical microscope (Carl Zeiss Axio Vert. A, Berlin, Germany) to perform a descriptive analysis of the changes in the configuration of the sulcus epithelium, gingival fibers, periodontal fibers (mainly crestal fibers), height, and alveolar crest integrity.

CAL was determined considering the distance between the cementoenamel junction (CEJ) and the closest point of the alveolar crest. Histometric analysis was performed using the ZEN program, 2 blue edition (Carl Zeiss Microscopy GmbH, 2011), drawing a line between both points on the buccal and palatal surfaces, based on what was reported by Semenoff.[37]

2.5 | Immunofluorescence

The expression of MMP-2 and MMP-13 was measured by the anti-MMP test with the indirect immunofluorescence (IIF) method. Briefly, the primary antibodies used were MMP-2 (K-20) (sc-8835) (Santa Cruz Biotechnology, Dallas, TX, USA), secondary antibodies Fluorescein (FITC)-conjugated AffiniPure Donkey anti-Goat IgG (H+L) (705–095-003 Jackson ImmunoResearch, West Grove, PA, USA), MMP-13 primary antibody (MMP-13 Antibody (NBP1-45723) Novus Biologicals, Denver, CO, USA), secondary antibody Rhodamine Red X-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (111-295-003 Jackson ImmunoResearch), and Hoechst 33342 solution (20 mM) (62 249 Thermo Fisher Scientific, Waltham, MA, USA). The slides were analyzed with a confocal microscope (Leica, SP2, Wetzlar, Germany) and observed at the junctional epithelium at 60×. Twenty ROIs were selected per area to determine the relative fluorescence intensity (RFI).

2.6 | Real-time quantitative PCR

Total RNA was extracted and purified from the right maxillae using a QIAcube and RNeasy Mini Kit (Qiagen, Venlo, the Netherlands)

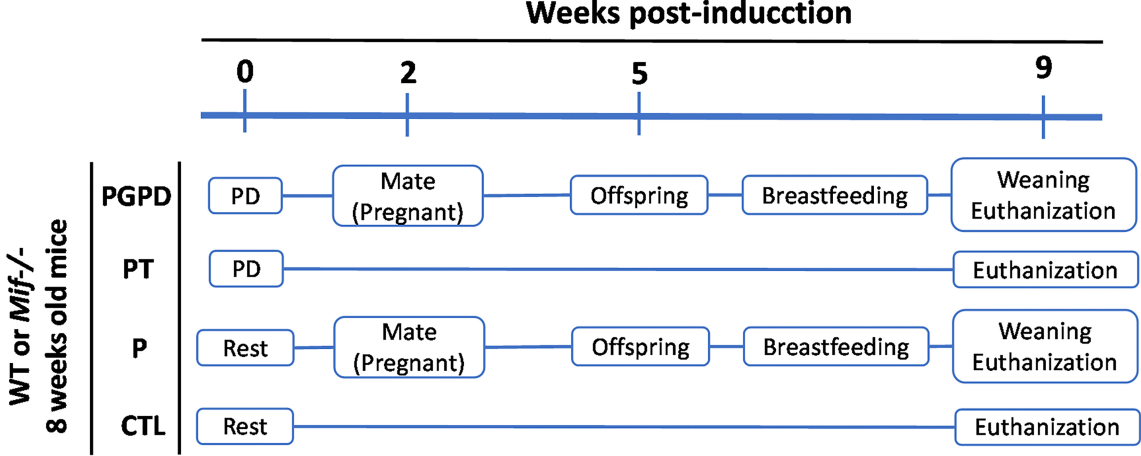


FIGURE 1 Experimental design. Six- to eight-week-old age-matched WT and *Mif-/-* female mice, both with a BALB/c genetic background, were grouped as follows: control (CTL), pregnant (P), periodontitis (PT), and pregestational periodontal disease (PGPD). The strategy for PGPD was as follows: induction: PD induction (time 0), 2 weeks later, mating (2 weeks post-induction), pregnancy development, and offspring (5 weeks post-induction); after lactation and weaning, all mice were euthanized (9 weeks post-induction). Three to five mice per group were used and two independent experiments were performed.

following the manufacturer's instructions. The RNA concentration was determined by measuring the absorbance at 260 nm using a Take3 plate in an Epoch Microplate Spectrophotometer (Agilent, Santa Clara, CA, USA). For the generation of complementary DNA (cDNA), total RNA was reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative PCR was performed using PowerSYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Relative gene expression values were normalized to the constitutive expression of β-actin. Specific primers for target genes are shown in Table 1 (all synthesized by Sigma-Aldrich).

2.7 | Statistical analysis

The analysis of the data obtained between the WT and *Mif-/-* groups was carried out using ANOVA, followed by Tukey's test for multiple comparisons and the Mann–Whitney U test. Values of $p < .05$ were considered statistically significant: (a) compared with healthy mice (CTL), (b) compared with pregnant group (P), (c) compared with periodontitis group (PT), (*) *Mif-/-* compared with WT. GraphPad Prism 8.0 software was used (Graph Pad Software, Inc., San Diego, CA, USA).

3 | RESULTS

3.1 | Periodontal tissue from mice with PGPD displayed significantly more MIF than that from mice with periodontitis

To analyze the influence of MIF on the exacerbation of preexisting periodontitis, we first quantified MIF in the systemic circulation of the WT and *Mif-/-* mice from all groups by ELISA. We did not observe significant differences between the WT groups, and the *Mif-/-* mice did not produce MIF at the systemic level (Figure S3). Thus, the differences in the production of MIF were appreciated locally in the periodontal tissue, as shown in Figure 2, transcripts in the periodontal tissue of healthy mice (CTL), pregnant mice (P), mice with periodontitis (PT), and pregnant mice with PGPD 9 weeks after periodontitis induction.

The P group of mice showed a significant increase in MIF in periodontal tissue compared to the CTL mice (1.04 vs 3.32 $^{a}p < .05$), while the PT group had a slight increase in the MIF transcripts compared to the CTL group (1.04 vs 1.75 $^{a}p < .05$). Importantly, tissues from PGPD mice showed a statistically significant increase compared to tissues from CTL and PT mice (1.04 and 1.75 vs 3.75 $^{a,c}p < .05$; Figure 2). MIF transcripts were not identified in *Mif-/-* mice.

TABLE 1 Sequences of primers used for gene expression analysis for RT-qPCR.

Primer	Sequence	T (°C)
mif F	5′-GGACCGGGTCTACATCAACT-3′	58
mif R	5′-CTCAAAGAACAGCGGTGCAG-3′	
ifn-γ F	5′-AGCGGCTGACTGAACTCAGATTGTAG-3′	57
ifn-γ R	5′-GTCACAGTTTTCAGCTGTATAGGG-3′	
tnf-α F	5′-GGCAGGTCTACTTTGGAGTCATTGC-3′	59
tnf-α R	5′-ACATTCGAGGCTCCAGTGAATTCGG-3′	
il-17 F	5′-AAAGCTCAGCGTGTCCAAAC-3′	60
il-17 R	5′-TGGAACGGTTGAGGTAGTCTG-3′	60
β-Actin F	5′-GTGACGTTGACATCCGTAAAGA-3′	60
β-Actin R	5′-GCCGGACTCATCGTACTCC-3′	

3.2 | *Mif-/-* mice develop attenuated clinical symptoms in pregestational periodontitis

We further investigated the biological impact of MIF observed in PT and pregnancy, combined and alone. We developed a PGPD model in *Mif-/-* and WT mice. We compared the clinical development of PT

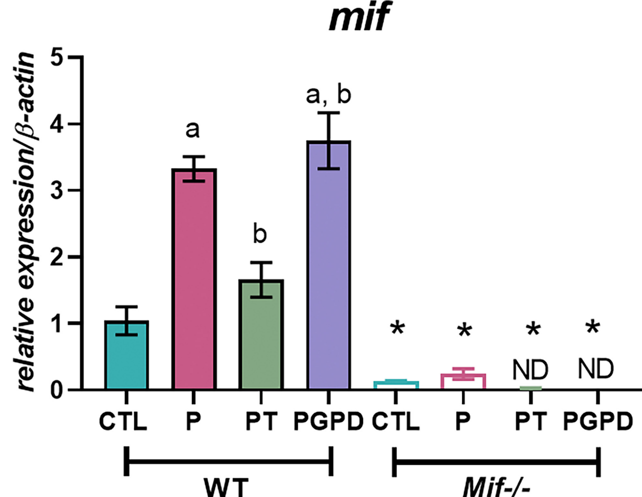


FIGURE 2 Periodontitis induces MIF expression, and pregnancy induces MIF overexpression in pregestational periodontal disease. Female mice with periodontitis (PT) and female mice with pregestational periodontitis (PGPD) were euthanized at 9 weeks post-induction of periodontitis; MIF expression in periodontal tissue was determined by RT-qPCR. Healthy (CTL) and pregnant (P) mice were used as controls. Data are expressed as the mean ± SEM and are representative of two independent experiments with at least three mice per group. Values of $p < .05$ were considered statistically significant (A) compared with healthy mice (CTL), (B) compared with the pregnant group (P), and (c) compared with the periodontitis group (PT) using ANOVA with Tukey's multiple comparison test. *Mif-/-* versus WT, (ND) not detected, using ANOVA with Tukey's multiple comparison test and Mann–Whitney *U* test.

at 9 weeks post-induction. As controls, healthy mice (CTL), pregnant mice (P), and periodontitis mice (PT) were used.

The maxillae from CTL WT and CTL *Mif-/-* mice exhibited normal characteristics with coral coloration, firmly adhered attached gingiva, and knife-edge free gingiva surrounding the molars, with no evidence of bleeding or inflammation (Figure 3A, CTL WT and CTL *Mif-/-*, respectively).

The tissues of the maxillae in WT PT mice presented the expected macroscopic signs of periodontal disease: severe inflammation, redness, loss of integrity, formation of periodontal pockets, and bleeding around the first molar (Figure 3B, WT PT). In contrast, we observed that the tissues of the PT *Mif-/-* group exhibited less inflammation and preserved tissue continuity (Figure 3B).

On the other hand, the P WT group showed tissues with slight redness on the gingival margin. Conversely, the tissues of the P *Mif-/-* group did not show apparent inflammatory changes, and their characteristics were comparable to those of the CTL groups (Figure 3C, P WT and P *Mif-/-*, respectively).

When PD was induced prior to gestation in both groups (WT and *Mif-/-*), we found an increase in the space of the gingival sulcus in the former group, whereas in the PGPD *Mif-/-* group, periodontal tissue was present with less inflammation, mild redness, and no visible lesions (Figure 3D, WT PGPD and *Mif-/-* PGPD, respectively).

3.3 | MIF deletion decreases periodontal tissue damage and reduces attachment loss in pregestational periodontitis

To confirm the clinical observations, a histometric analysis was performed, measuring the CAL in the periodontal tissue of the right second molar. The CTL WT and CTL *Mif-/-* groups showed normal histological characteristics, similar between both groups: the epithelium of the sulcus and alveolar crest, the insertion of connective tissue fibers, gingival fibers, the junctional epithelium, and Sharpey's fibers, in their insertion to the alveolar bone and root cementum, were intact and presented a constant and symmetrical thickness, as well as the cell nuclei in the PDL fibers and the osteocytes of the alveolar bone (Figure 4A, CTL WT and CTL *Mif-/-*, 10×, 40×). CAL depth was very similar between the CTL WT and *Mif-/-* groups for both buccal and palatal surfaces (113 ± 3 vs 127 ± 3 nm and 140 ± 3 vs 131 ± 4 nm; Figure 4B,C, respectively).

The PT WT group presented severe damage to the periodontal tissues, with apical migration of the epithelium and formation of periodontal pockets, loss of the alveolar crest (gray arrow), detachment of Sharpey's fibers on the surface of the root cementum, and the absence of cell nuclei, characteristic of the destruction of periodontal tissues (Figure 4A, PT WT 10×, 40×). Interestingly, the PT

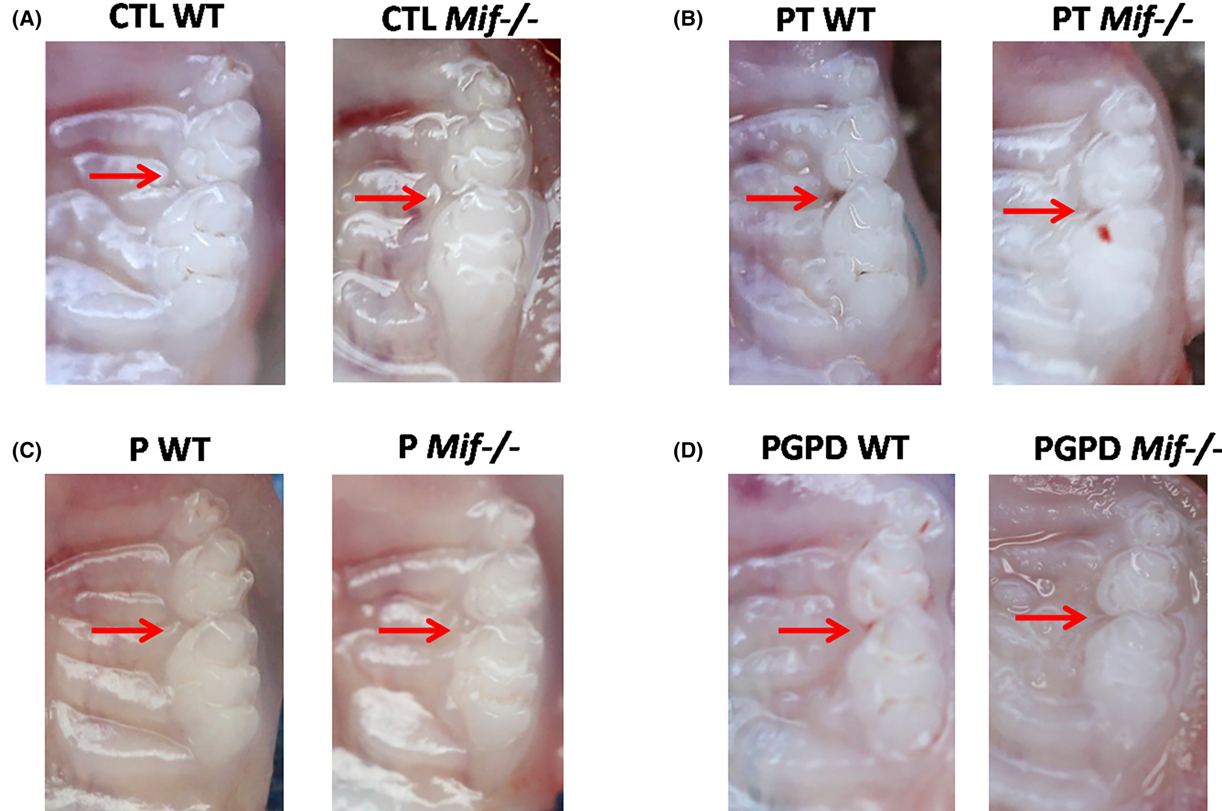


FIGURE 3 *Mif-/-* mice develop attenuated clinical symptoms in pregestational periodontal disease. Clinical photographs of periodontal tissues in groups (A) CTL WT and *Mif-/-*, (B) PT WT and *Mif-/-*, (C) P WT and *Mif-/-*, (D) PGDP WT and *Mif-/-*, after the induction of periodontitis (PT), around the upper right second molar. The red arrow indicates the interproximal region and the palatal gingiva. The images are representative of three independent experiments with $n = 3$.

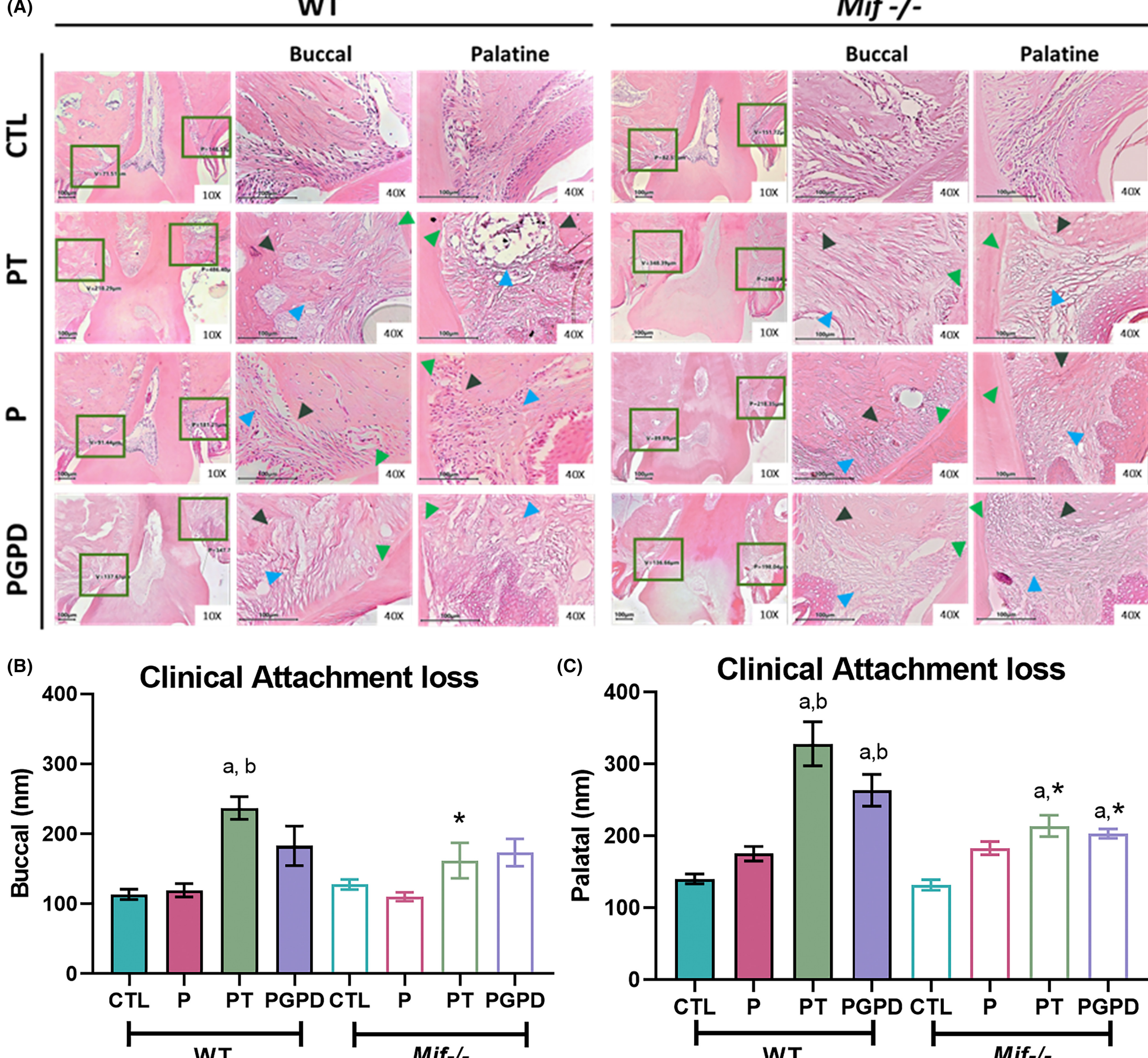


FIGURE 4 *Mif-/-* mice show lower periodontal tissue damage and attachment loss than wild-type mice in the pregestational periodontitis model. (A) Representative H&E-stained histology sections, CAL measurement shown, by drawing a line from the CEJ to the bone crest. First panel 10×, marked in the green box 40× panel, on the left buccal/vestibular (V) and right palatal (P) surfaces, WT vs *Mif-/-*. Arrowheads: alveolar crest loss (gray), sulcus epithelium migration (blue), Sharpey's fibers insertion (green). Histometric analysis of the clinical attachment loss. (B) Buccal, (C) Palatine. Groups CTL WT, PT WT, P WT and PGPD WT, CTL *Mif-/-*, PT *Mif-/-*, G *Mif-/-*, and PGPD *Mif-/-*. A representative image of three independent experiments is shown. Values of $p < .05$ were considered statistically significant, (A) compared with healthy mice (CTL), (B) compared with pregnant group (P), and (C) compared with periodontitis group (PT), (*) *Mif-/-* versus WT, using ANOVA with Tukey's multiple comparison test and Mann–Whitney *U* test.

Mif-/- group had changes in the distribution of gingival fibers, less loss of continuity was observed, and Sharpey's fibers remained adhered to the root cementum (blue arrow), although they increased in thickness compared to the PT WT group (Figure 4A, PT *Mif-/-*, 10×, 40×, green arrow). In addition, the lower tissue damage of the PT *Mif-/-* group was associated with lower CAL on the buccal and palatal aspects compared to the PT WT group (237 ± 8 vs 161 ± 36 nm and 325 ± 15 vs 213 ± 2, *$p < .05$; Figure 4B,C).

In the PGPD WT group, periodontal pockets were observed due to attachment loss on the buccal and palatal side, the deepest on the palatal side (Figure 4A, PGPD WT, 10×, 40×), with loss of continuity in the periodontal tissues, detachment of Sharpey's fibers from the root cementum, and absence of cell nuclei (Figure 4A, PGPD WT, 10×, 40×, gray arrow). Importantly, the palatal aspect of the PGPD *Mif−/−* group developed less damage than that of the PGPD WT group. The PGPD *Mif−/−* group

presented intact gingival sulcus, bone crest, and junctional epithelium (Figure 4A, PGPD *Mif-/-*, 10×, 40×, gray arrow). Additionally, the PGPD *Mif-/-* group presented the insertion of connective tissue fibers, gingival fibers, and the PDL with changes in their distribution and configuration, without loss of continuity. Sharpey's fibers were found adhered to the root cementum and alveolar bone, although they were slightly thicker than those in the PGPD WT group (Figure 4A, PGPD *Mif-/-*, 10×, 40×, blue arrow). The CAL of the PGPD *Mif-/-* group was significantly lower than the CAL of the PGPD WT group (263 ± 32 vs 203 ± 3 nm, *$p < .05$; Figure 4B,C). It should be noted that the vestibular wall is much thicker than the palatal wall in mice, unlike in humans, in which the vestibular wall is the thinnest, and sometimes, it does not exist.[38]

3.4 | *Mif-/-* mice display rearranged collagen fibers

Collagen is one of the most important components of periodontal tissues, mainly PDL, which is made up mostly of type I and III collagen and to a lesser extent of type IV, V, VII, and XII collagen.[39] Due to the changes in the configuration of the periodontal tissues, we decided to perform Masson's staining to observe changes in the distribution of collagen and keratin.

The distribution and organization of collagen in the connective tissue of the alveolar bone, PDL, and Sharpey's fibers in both the alveolar and cemental surfaces of the CTL WT and CTL *Mif-/-* groups presented very similar organization (Figure 5). The PT WT group showed a decrease in the distribution and amount of collagen with migration of keratin into the periodontal pocket (Figure 5, 10×, 40× green arrow), crest loss (gray arrow), and separation of collagen fibers (Figure 5, 10×, 40× yellow arrow).

PT *Mif-/-* mice showed changes in collagen distribution in the gingival fibers, which changed their position surrounding the ligation area without destruction or structural loss (Figure 5, 10×, 40×, yellow arrow). Additionally, the PDL fibers and Sharpey's fibers were observed to be thickened, with a greater presence of collagen compared to the PT WT group (Figure 5, 10×, 40×, red arrow).

The P *Mif-/-* group presented a higher proportion of collagen in the PDL and in Sharpey's fibers than the P WT group (Figure 5, 10×, 40× red arrow). In line with this, the PGPD *Mif-/-* group presented widened Sharpey fibers, with dense collagen, firmly attached to the root cementum (Figure 5, 10×, 40× red and yellow arrow). The PGPD WT group showed loss of the crest (gray arrow), apical migration of connective tissue mainly on the palatal side, destruction of periodontal tissues, detachment of Sharpey's fibers, and loss of organization of the collagen fibers of the PDL (yellow arrow) (Figure 5, 40×).

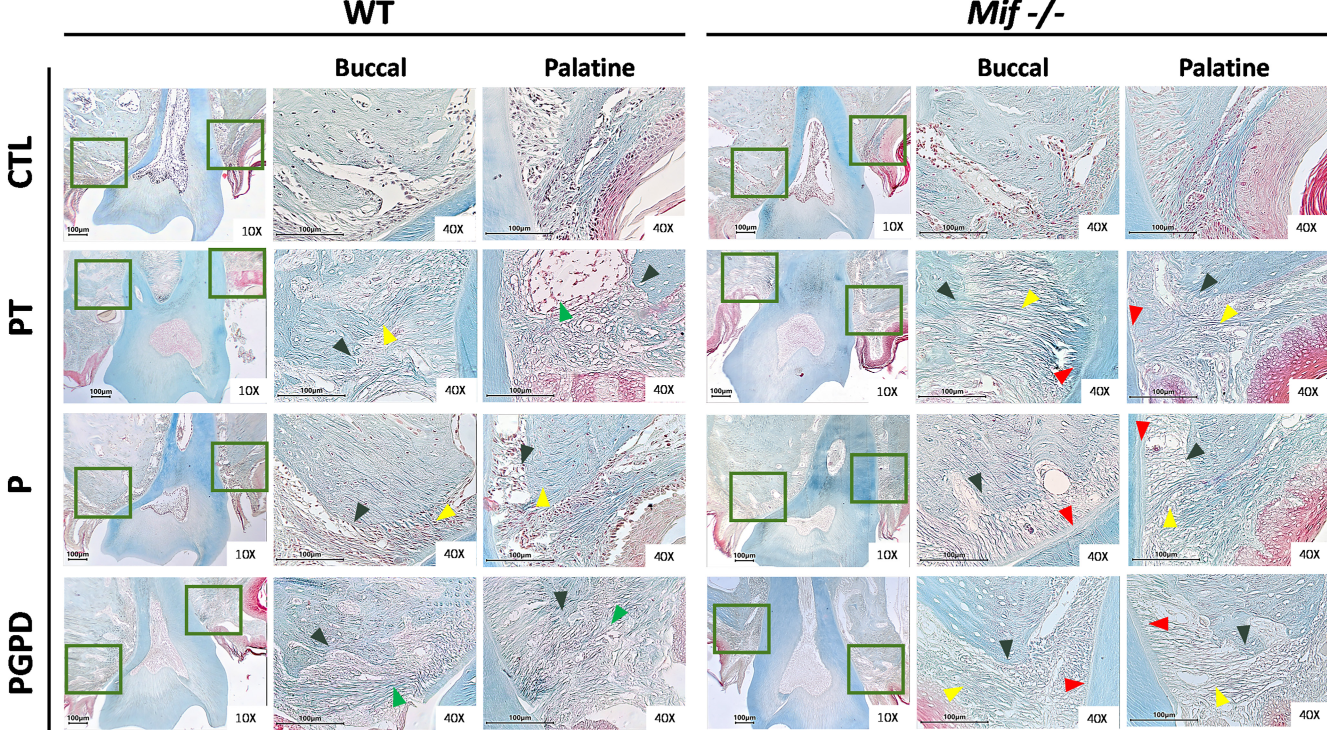


FIGURE 5 MIF deletion favors the preservation of collagen and periodontal pocket epithelium. Representative histological sections stained with Masson trichrome, showing the distribution of collagen in periodontal tissues. First panel 10×, marked in the green box 40×, on the left buccal/vestibular (V) and right palatal (P) surfaces, WT vs *Mif-/-*. To identify collagen (blue), keratin (red) and nuclei (black), keratin and epithelium (red), B (bone), L (periodontal ligament), G (gingiva), arrow heads: alveolar crest loss (gray), keratin migration (green), collagen enlargement (red), gingival fiber distribution (yellow). Groups CTL WT, PT WT, P WT and PGPD WT, CTL *Mif-/-*, PT *Mif-/-*, P *Mif-/-*, and PGPD *Mif-/-*. A representative image of three independent experiments is shown, $n = 6$.

3.5 | Pregestational periodontitis-induced in *Mif-/-* mice show downregulation of MMP-2 and MMP-13 expression during pregnancy

Due to the observed changes in the distribution of collagen fibers in our model, we decided to determine the relative fluorescence expressed by the most important cell matrix-degrading metalloproteinases, MMP-2 and MMP-13.

The P WT and PT WT groups presented increased expression of MMP-2, mainly in the PDL (Figure 6B,C), and this expression was statistically significant compared to the CTL WT (Figure 6A) group (306.48 ± 16 vs 652.13 ± 15 and 663.63 ± 3 RFI, $^{a}p < .05$; Figure 6E). The PGPD WT group did not show significant changes in MMP-2 expression compared to the WT CTL group (Figure 6A,E).

Importantly, the PT *Mif-/-* group was the only group that presented significant expression of MMP-2 compared to the *Mif-/-* CTL group (321.59 ± 6 vs 549.51 ± 3 RFI $^{a,}*p < .05$; Figure 6A,E). Notably, the *Mif-/-* PT group expressed significantly less MMP-2 than the WT PT group (663.63 ± 3 vs 549.51 ± 3 RFI, $*p < .05$; Figure 6B,F).

On the other hand, only the P WT and PGPD WT groups presented a statistically higher expression of MMP-13 compared to the CTL WT group (349.01 ± 2 vs 839.5 ± 8 vs 640.19 ± 2 RFI, $^{a}p < .05$; Figure 6A,C,F). Interestingly, in the absence of MIF, only the P *Mif-/-* group presented a slightly statistically significant increase (546.4 ± 2 vs 642.7 ± 8 RFI, $^{a}p < .05$) in the expression of MMP-13 with respect to the CTL group *Mif-/-* (Figure 6A,F). It should be noted that the basal expression of MMP-13 was slightly higher in the CTL *Mif-/-* group than in the CTL WT group.

3.6 | Periodontitis-induced *Mif-/-* mice develop lower levels of inflammatory cytokines

It is well recognized that MIF stimulates the expression and secretion of proinflammatory cytokines such as TNF-α and IFN-γ. Since overexpression of MIF was observed in the P WT and PGPD WT groups, we quantified the *tnf-α*, *inf-γ*, and *il-17* transcripts in the periodontal tissue of all groups to establish their relative expression in the exacerbation of gestational periodontitis.

The expression of *tnf-α* was significantly elevated in the P WT, PT WT, and PGPD WT groups compared to the control (1.03 vs 9.12 vs 10.83 vs 12.07 $^{a}p < .05$). The P *Mif-/-*, PT *Mif-/-*, and PGPD *Mif-/-* groups expressed significantly less *tnf-α* than the P WT, PT WT, and PGPD WT groups (9.12 vs 0.92, 10.83 vs 0.82 $*p < .05$; Figure 7A).

Regarding the expression of the *inf-γ* transcript, it was observed that the P WT, PT WT, and PGPD WT groups showed reduced expression with respect to the CTL WT group (1.00 vs 0.26 vs 0.36 vs 0.56 $^{a}p < .05$). Importantly, the WT PGPD group expressed significantly more *inf-γ* than the P WT group (0.56 vs 0.36 $^{b}p < .05$; Figure 7B). The *Mif-/-* groups expressed significantly less *inf-γ* in all groups, P *Mif-/-*, PT *Mif-/-*, and PGPD *Mif-/-*, compared to their respective WT groups (1.00 vs 0.05, 0.26 vs 0.072, 0.36 vs 0.09, and 0.56 vs not detected, $*p < .05$; Figure 7B).

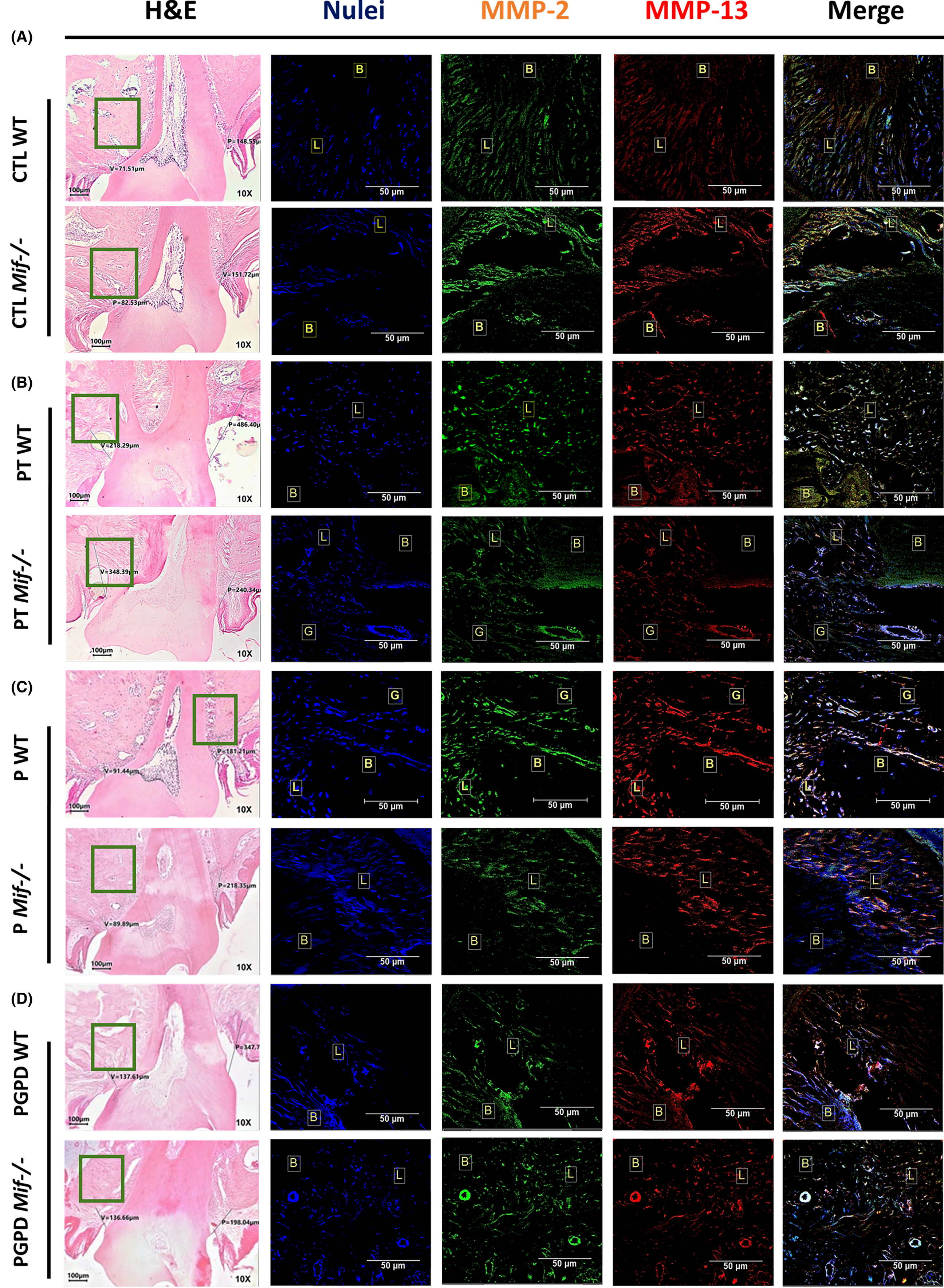
The expression of the *il-17* transcript was significantly increased in the PT WT group compared to the CTL WT, P WT, and PGPD WT groups (1.03, 1.50 vs 45.73 $^{a,b}p < .05$; Figure 7C). However, the PGPD *Mif-/-* group presented significantly more *il-17* than the CTL *Mif-/-*, P *Mif-/-*, and PT *Mif-/-* groups (72.94 vs 8.69, 4.90, 28.34 $^{a,b,c}p < .05$; Figure 7C).

4 | DISCUSSION

MIF is significantly overexpressed in periodontal tissues and crevicular fluid in periodontitis,[40,41] and it has also been reported to be overexpressed during pregnancy in gestational tissues.[24,30,41] However, the role of MIF during the development and/or aggravation of periodontitis in pregnancy is unknown. Therefore, here, we used WT and *Mif-/-* mice to study the influence of the expression of gestational MIF on the aggravation of periodontitis existing before pregnancy. We identified an increase in MIF transcripts in the periodontal tissues in PT, a higher increase in P mice, and an even higher increase when both conditions were combined, PGPD.[28,42] Interestingly, the increase was maintained until the end of pregnancy (P WT) and persisted up to 4 weeks after the weaning of the pups.

To identify how these increases in MIF affected the development of PT, we analyzed clinical and histological data in groups of *Mif-/-* and WT mice.[43] The gingival margins in which we identified characteristics present in periodontitis were the PT WT and PGPD WT groups. The PT *Mif-/-* and PGPD *Mif−/−* groups did not develop clinical signs and preserved tissue continuity compared to the PT WT and PGPD WT groups. These observations were corroborated by a histometric analysis[44,45] and supported that the presence of MIF influences the exacerbation of PT during pregnancy. In particular, the loss of tissue from the PGPD WT and PT WT groups was similar, with no significant differences, although both showed tissue destruction, mainly on the palatal side, with a 31% greater loss than on the buccal side. This probably because the buccal wall

FIGURE 6 Pregestational periodontitis-induced *Mif-/-* mice show downregulation of MMP-2 and MMP-13 expression during pregnancy. Immunofluorescence of MMP-2 (green), MMP13 (red), Hotch (nuclei, Blue) present in the periodontal tissue, at the level of the bone crest, indicated in the box, to identify: B (bone), L (periodontal ligament), G (gingiva). (A) CTL WT and *Mif-/-*, (B) PT WT and *Mif-/-*, (C) P WT and *Mif-/-*, (D) PGDP WT and *Mif-/-*, (E) MMP-2 relative expression, and (F) MMP-13 relative expression. A representative image of two independent experiments is shown. The images were observed at 63×. A representative image of three independent experiments is shown. Values of $p < .05$ were considered statistically significant, (a) compared with healthy mice (CTL), (b) compared with pregnant group (P), and (c) compared with periodontitis group (PT), (*) *Mif-/-* vs WT, using ANOVA with Tukey's multiple comparison test and Mann–Whitney *U* test.


H&E
Nulei
MMP-2
MMP-13
Merge
(A)
CTL WT
CTL Mif-/-
(B)
PT WT
PT Mif-/-
(C)
P WT
P Mif-/-
(D)
PGPD WT
PGPD Mif-/-
10X
100µm
50 µm
B
L
G

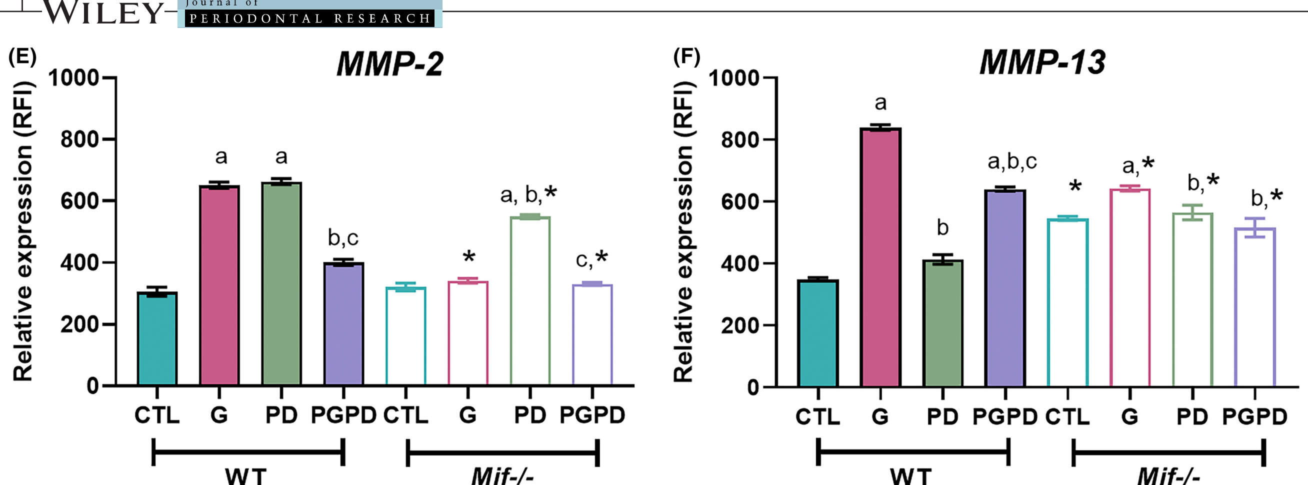


FIGURE 6 (Continued)

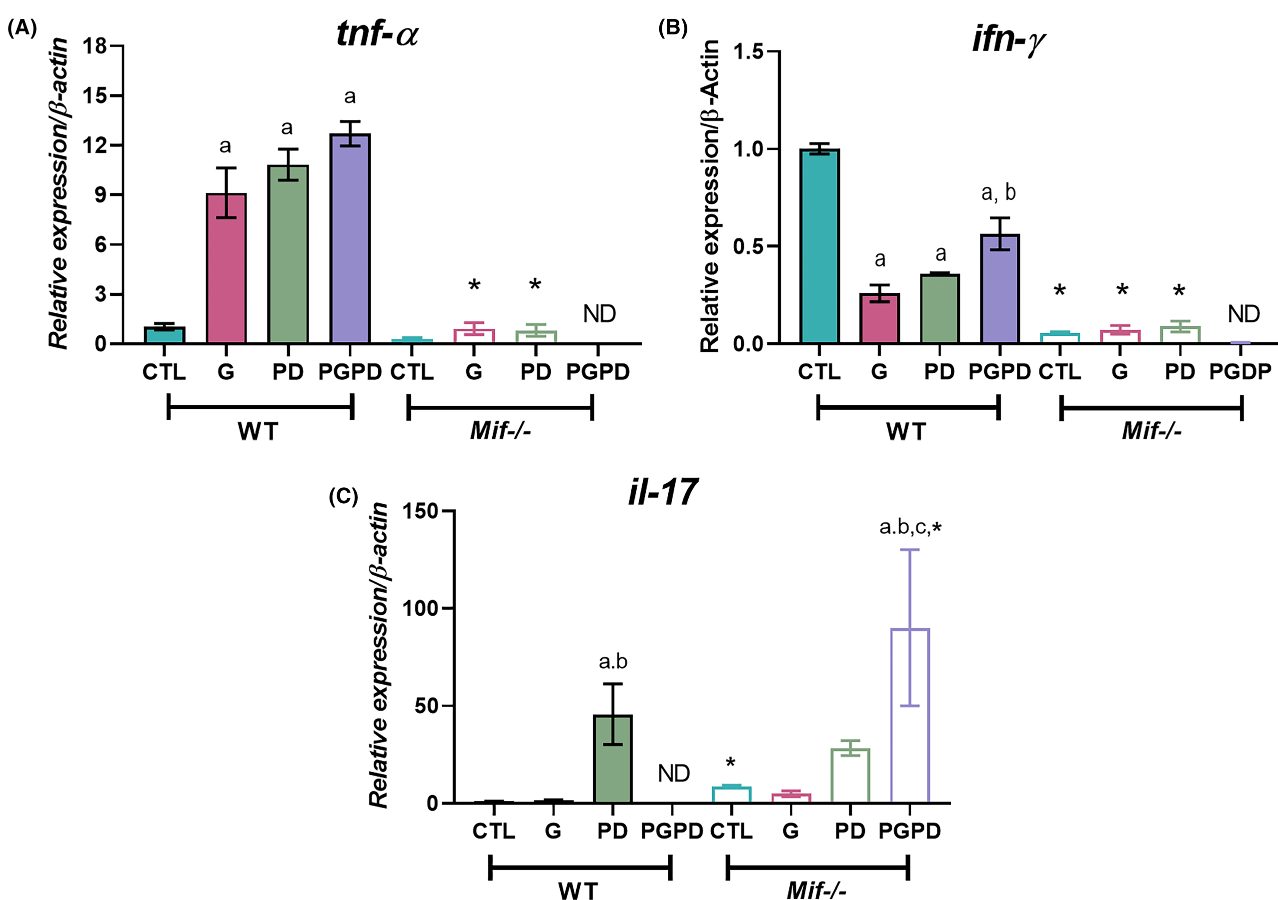


FIGURE 7 Deletion of MIF abates the expression of *tnf-α* and *ifn-γ* but not the expression of *il-17* in periodontitis. Female mice with periodontitis (PT) and female mice with pregestational periodontitis (PGPD) were euthanized at 9 weeks post-induction of periodontitis; MIF expression in periodontal tissue was determined by RT-qPCR. Healthy (CTL) and pregnant (P) mice were used as controls. Data are expressed as the mean ± SEM and are representative of two independent experiments with at least three mice per group. Values of $p < .05$ were considered statistically significant, (A) compared with healthy mice (CTL), (B) compared with pregnant group (P), and (C) compared with periodontitis group (PT), (*) *Mif-/-* vs WT, (ND) Not Detected, using ANOVA with Tukey's multiple comparison test and Mann–Whitney U test.

is wider in mice opposite to humans, in which the buccal wall is thinner.[45]

The results described above were not consistent with Hays et al., who reported greater depth in the pregnancy and periodontitis groups than in the periodontitis-only group. This discrepancy may be because they established their periodontitis model 8 days after pregnancy confirmation,[46] while in the study reported here, periodontitis was induced 2 weeks prior to pregnancy. Additionally, here,

the transition to lactation was allowed, which lasted 4 weeks after delivery, a period in which there could be recovery of the damaged periodontal tissue, as occurs in humans.[47]

In the *Mif*−/− mice, the PT and PGPD groups, presented less damage, and the gingival sulcus, the bone crest, and the epithelium were conserved, with little loss of continuity in the periodontal tissues, and less detachment of Sharpey's fibers than the PT and PGPD WT groups.[27] Taken together, the results suggest that the expression of MIF favors the damage associated with periodontitis during pregnancy.

The above assertion was confirmed by observations of the distribution of collagen. In the PT *Mif*−/− and PGPD *Mif*−/− groups, we report for the first time, to our knowledge, the reconfiguration in both the direction of the collagen fibers and the widening of the Sharpey and gingival fibers in *Mif*−/− mice. This reconfiguration in the distribution of the fibers could be related to early stages of fibrosis, since fibroblasts during the proliferation phase can express extracellular matrix, specifically collagen in the form of scars.[48] In line with this, tissue remodeling is highly dependent on collagenase and metalloproteinase enzymes that degrade extracellular collagen. Thus, we measured the presence of MMP-2 and MMP-13, which degrade type IV, V, VII, X, XI, and XII collagen, fibronectin, and elastin,[49] and MMP-13 degrades collagen I, II, III, IV, proteoglycans, fibronectin, fibrin, and tenasin,[50] respectively. We identified lower MMP-2 expression in the PT *Mif*−/− and P *Mif*−/− groups.[51]

The aforesaid observations reinforce that the presence of MIF favors the expression of MMP-2, which is associated with the greater destruction of periodontal tissues present in the WT groups, but importantly, our results also indicate that MIF is not the only inducer of MMP-2 expression, since the PT *Mif*−/− group overexpressed MMP-2, although to a lesser extent than the PT WT group, supporting that there are other factors contributing to this effect. MMP-2 in PT *Mif*−/− mice could participate in remodeling, rather than tissue destruction; however, other studies are necessary to deepen our understanding of its participation.

Moreover, a significant increase in MMP-13 was observed in the P WT and PGPD WT groups, as well as a moderate increase in this enzyme in the P *Mif*−/− group, which suggests that gestation may be a determinant for the expression of MMP-13 and possibly exacerbation of PT. Importantly, we did not identify an overexpression of MMP-13 in the PT WT group, contrary to what was previously reported in human PT patients.[51] This inconsistency may be inherent to the mouse model used or the methodology, since the western blot technique used by Ejil et al. may be more sensitive than the method used in this study.

Finally, we identified elevated concentrations of *tnf-α* and *il-17* in the PT WT group, consistent with the loss of tissue integrity characteristic of periodontal disease, as previously reported by Mesa et al., in patients with PT.[52] Regarding the P WT and PGPD WT groups, we identified overexpression of *tnf-α* and *mif*. These results suggest that pregnancy favors the exacerbation of periodontitis associated with inflammatory cytokines such as TNF-α and MIF. Regarding *tnf-α*, our results contrast with previous reports that showed that tissues from pregnant patients with PT expressed lower levels of *tnf-α* than tissues from healthy individuals.[53,54]

Interestingly, MIF absence decreased *tnf-α* and *ifn-γ* transcripts in the PT *Mif*−/− group, while *il-17* was overexpressed (although at lower levels than the PT WT group), which may be because the tissues of the PT *Mif*−/− group presented tissue remodeling, which could be associated with the maintenance of epithelial integrity promoted by IL-17.[55]

Immune responses depend on the interaction with the microbiome, which releases virulence factors that interact with a wide variety of cells, such as the development and progression of periodontal disease.[56] MIF is involved in the upregulation of TLR-4,[19] a pattern recognition receptor that recognizes lipopolysaccharides, a component of gram negative bacteria, which favors monocyte, Mφ, and dendritic cell (DC) activation.[20] Establishing this interaction may help to understand the MIF-pregnancy role in PT development.

One factor that influences the composition of the microbiota is the hormonal profile that is affected in contraceptive users and pregnant women,[6,57] which supports the assumption that hormonal load contributes to periodontal disease by altering the microbiome.[58] Therefore, it would be interesting to analyze the profiles of cytokines and proinflammatory hormones at the beginning, middle, and end of gestation to better understand the effect of pregnancy on the exacerbation of periodontitis.

5 | CONCLUSIONS

Our data demonstrate that MIF exacerbates pregestational periodontitis favoring the overexpression of TNF-α and MMP-13 at the end of pregnancy. Deletion of MIF demonstrates that cytokine has a strong influence on the aggravation of preexisting periodontal disease during pregnancy, since *Mif*−/− pregnant mice developed less severe disease than WT pregnant mice. In addition, we also demonstrated that MIF regulates TNF-α release and MMP-13 expression, and both contribute to the development of the disease.

AUTHOR CONTRIBUTIONS

Betsaida J. Ortiz-Sánchez performed conception, acquisition of data, analysis, drafting the article, final approval, Imelda Juárez-Avelar, acquisition of data, analysis and interpretation, revising draft, and final approval. Antonio Andrade-Meza involved in interpretation of data, revising draft, and final approval. Mónica Gabriela Mendoza-Rodríguez performed analysis and interpretation of data, revising draft, and final approval. Yolanda I. Chirino involved in acquisition of data, revising draft, and final approval. Eric Monroy-Pérez involved in acquisition of data, revising draft, and final approval. Gloria Luz Paniagua-Contreras performed analysis and interpretation of data, revising draft, and final approval. Miriam Rodriguez-Sosa involved in conception and design, analysis and interpretation of data, revising draft, and final approval.

All authors read and approved the manuscript and agreed to the submission of the manuscript to the journal.

ACKNOWLEDGEMENTS

Betsaida Julieta Ortiz-Sánchez is a doctoral student from the Programa de Doctorado en Ciencias Biológicas, Universidad Nacional Autónoma de México (UNAM), and received a fellowship from the Council for Science and Technology of the State of Mexico (COMECyT; 2019-2020).

FUNDING INFORMATION

This research was funded in part by the Council for Science and Technology of the State of Mexico (COMECYT) grant number (FICDTEM-2021-072) and the Support Program for Research Projects and Technological Innovation (PAPIIT)-UNAM, grant number (IN-217021).

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Betsaida J. Ortiz-Sánchez https://orcid.org/0000-0003-4313-8854
Imelda Juárez-Avelar https://orcid.org/0000-0002-0504-6812
Antonio Andrade-Meza https://orcid.org/0009-0006-6338-0591
Mónica Gabriela Mendoza-Rodríguez https://orcid.org/0000-0003-3056-9997
Yolanda I. Chirino https://orcid.org/0000-0001-6627-3392
Eric Monroy-Pérez https://orcid.org/0000-0001-8028-424X
Gloria Luz Paniagua-Contreras https://orcid.org/0000-0003-2935-6177
Miriam Rodriguez-Sosa https://orcid.org/0000-0003-2911-9390

REFERENCES

1. Papapanou PN, Sanz M, Buduneli N, et al. Periodontitis: consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Periodontol*. 2018;89(Suppl 1):S173-S182.
2. Wu L, Zhang SQ, Zhao L, Ren ZH, Hu CY. Global, regional, and national burden of periodontitis from 1990 to 2019: results from the Global Burden of Disease study 2019. *J Periodontol*. 2022;93:1445-1454.
3. Tonetti MS, Jepsen S, Jin L, Otomo-Corgel J. Impact of the global burden of periodontal diseases on health, nutrition and wellbeing of mankind: a call for global action. *J Clin Periodontol*. 2017;44(5):456-462.
4. Duque A. Prevalencia de periodontitis crónica en Iberoamérica. *Rev Clin Periodoncia Implantol Rehabil Oral*. 2016;9(2):208-215.
5. Shiau HJ. Periodontal disease in women and men. *Curr Oral Health Rep*. 2018;5(4):250-254.
6. Balan P, Chong YS, Umashankar S, et al. Keystone species in pregnancy gingivitis: a snapshot of Oral microbiome during pregnancy and postpartum period. *Front Microbiol*. 2018;9:2360.
7. La Rocca C, Carbone F, Longobardi S, Matarese G. The immunology of pregnancy: regulatory T cells control maternal immune tolerance toward the fetus. *Immunol Lett*. 2014;162(1 Pt A):41-48.
8. Webb DA, Mathew L, Culhane JF. Lessons learned from the Philadelphia Collaborative Preterm Prevention Project: the prevalence of risk factors and program participation rates among women in the intervention group. *BMC Pregnancy Childbirth*. 2014;14:368.
9. Parachuru V, Coates D, Milne T, Rich A, Seymour G. FoxP3+ regulatory T cells, interleukin 17 and mast cells in chronic inflammatory periodontal disease. *J Periodontal Res*. 2018;53(4):622-635.
10. Di Benedetto A, Gigante I, Colucci S, Grano M. Periodontal disease: linking the primary inflammation to bone loss. *Clin Dev Immunol*. 2013;2013:503754.
11. Beklen A, Ainola M, Hukkanen M, Gürgan C, Sorsa T, Konttinen YT. MMPs, IL-1, and TNF are regulated by IL-17 in periodontitis. *J Dent Res*. 2007;86(4):347-351.
12. Luchian I, Goriuc A, Sandu D, Covasa M. The role of matrix metalloproteinases (MMP-8, MMP-9, MMP-13) in periodontal and Peri-implant pathological processes. *Int J Mol Sci*. 2022;23(3):1806.
13. Bucala R. MIF rediscovered: cytokine, pituitary hormone, and glucocorticoid-induced regulator of the immune response. *FASEB J*. 1996;10(14):1607-1613.
14. Fang T, Liu L, Song D, Huang D. The role of MIF in periodontitis: a potential pathogenic driver, biomarker, and therapeutic target. *Oral Dis*. 2023:1-17.
15. Baugh JA, Bucala R. Macrophage migration inhibitory factor. *Crit Care Med*. 2002;30(1):S27-S35.
16. Sumaiya K, Langford D, Natarajaseenivasan K, Shanmughapriya S. Macrophage migration inhibitory factor (MIF): a multifaceted cytokine regulated by genetic and physiological strategies. *Pharmacol Ther*. 2022;233:108024.
17. Flaster H, Bernhagen J, Calandra T, Bucala R. The macrophage migration inhibitory factor-glucocorticoid dyad: regulation of inflammation and immunity. *Mol Endocrinol*. 2007;21(6):1267-1280.
18. Calandra T, Bernhagen J, Mitchell RA, Bucala R. The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. *J Exp Med*. 1994;179(6):1895-1902.
19. Roger T, David J, Glauser MP, Calandra T. MIF regulates innate immune responses through modulation of toll-like receptor 4. *Nature*. 2001;414(6866):920-924.
20. Ruiz-Rosado JD, Olguin JE, Juarez-Avelar I, et al. MIF promotes classical activation and conversion of inflammatory Ly6C(high) monocytes into TipDCs during murine toxoplasmosis. *Mediators Inflamm*. 2016;2016:9101762.
21. Roger T, Glauser MP, Calandra T. Macrophage migration inhibitory factor (MIF) modulates innate immune responses induced by endotoxin and gram-negative bacteria. *J Endotoxin Res*. 2001;7(6):456-460.
22. Li X, Lan H, Huang X, Zhang C, Jin L. Expression profile of macrophage migration-inhibitory factor in human gingiva and reconstituted human gingival epithelia stimulated by Porphyromonas gingivalis lipopolysaccharide. *J Periodontal Res*. 2013;48(4):527-532.
23. Nonnenmacher C, Helms K, Bacher M, et al. Effect of age on gingival crevicular fluid concentrations of MIF and PGE2. *J Dent Res*. 2009;88(7):639-643.
24. Ortiz-Garcia YM, Garcia-Iglesias T, Morales-Velazquez G, et al. Macrophage migration inhibitory factor levels in gingival Crevicular fluid, saliva, and serum of chronic periodontitis patients. *Biomed Res Int*. 2019;2019:7850392.
25. Alhammadi A, Koippallil Gopalakrishnan AR, Saqan R, Badran Z, Al Kawas S, Rahman B. Salivary macrophage chemokines as potential biomarkers of gingivitis. *BMC Oral Health*. 2023;23(1):77.

26. Morimoto T, Nishihira J, Kohgo T. Immunohistochemical localization of macrophage migration inhibitory factor (MIF) in human gingival tissue and its pathophysiological functions. *Histochem Cell Biol*. 2003;120(4):293-298.
27. Madeira MFM, Queiroz-Junior CM, Costa GM, et al. MIF induces osteoclast differentiation and contributes to progression of periodontal disease in mice. *Microbes Infect*. 2012;14(2):198-206.
28. Ietta F, Bechi N, Romagnoli R, et al. 17β-estradiol modulates the macrophage migration inhibitory factor secretory pathway by regulating ABCA1 expression in human first-trimester placenta. *Am J Physiol Endocrinol Metab*. 2010;298(3):E411-E418.
29. Ietta F, Ferro EAV, Bevilacqua E, Benincasa L, Maioli E, Paulesu L. Role of the macrophage migration inhibitory factor (MIF) in the survival of first trimester human placenta under induced stress conditions. *Sci Rep*. 2018;8(1):1-10.
30. Ietta F, Todros T, Ticconi C, et al. Macrophage migration inhibitory factor in human pregnancy and labor. *Am J Reprod Immunol*. 2002;48(6):404-409.
31. Rose L, Genco R, Cohen W. Periodontal medicine. *Periodontal Medicine and the Female Patient*. BC Decker Inc Canada; 2000:162-165.
32. Ortiz-Sánchez BJ, Legorreta-Herrera M, Rodriguez-Sosa M. Influence of gestational hormones on the bacteria-induced cytokine response in periodontitis. *Mediators Inflamm*. 2021;2021:5834608.
33. Bozza M, Satoskar AR, Lin G, et al. Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis. *J Exp Med*. 1999;189(2):341-346.
34. Laird PW, Zijderveld A, Linders K, Rudnicki MA, Jaenisch R, Berns A. Simplified mammalian DNA isolation procedure. *Nucleic Acids Res*. 1991;19(15):4293.
35. Benzen B, Grauballe MCB, Björnsson M, Stoltze K, Hjørting-Hansen E, Holmstrup P. A comparison of two models of experimental periodontitis in rats. *Scand J Lab Anim Sci*. 2005;32(2):73-80.
36. Abe T, Hajishengallis G. Optimization of the ligature-induced periodontitis model in mice. *J Immunol Methods*. 2013;394(1–2):49-54.
37. Semenoff TA, Semenoff-Segundo A, Bosco AF, Nagata MJ, Garcia VG, Biasoli ER. Histometric analysis of ligature-induced periodontitis in rats: a comparison of histological section planes. *J Appl Oral Sci*. 2008;16(4):251-256.
38. Zhang X, Li Y, Ge Z, Zhao H, Miao L, Pan Y. The dimension and morphology of alveolar bone at maxillary anterior teeth in periodontitis: a retrospective analysis-using CBCT. *Int J Oral Sci*. 2020;12(1):4.
39. Nanci A. *Ten Cate's Oral Histology-e-Book: Development, Structure, and Function*. Elsevier Health Sciences; 2017.
40. Gürkan A, Eren G, Çetinkalp Ş, Akçay YD, Emingil G, Atilla G. Monocyte chemotactic protein-1, RANTES and macrophage migration inhibitory factor levels in gingival crevicular fluid of metabolic syndrome patients with gingivitis. *Arch Oral Biol*. 2016;69:82-88.
41. Zhang D, Xu T, Xu Q, et al. Expression profile of macrophage migration inhibitory factor in periodontitis. *Arch Oral Biol*. 2021;122:105003.
42. Paulesu L, Bhattacharjee J, Bechi N, Romagnoli R, Jantra S, Ietta F. Pro-inflammatory cytokines in animal and human gestation. *Curr Pharm Des*. 2010;16(32):3601-3615.
43. Liberman DN, Pilau RM, Orlandini LF, Gaio EJ, Rösing CK. Comparison of two methods for alveolar bone loss measurement in an experimental periodontal disease model in rats. *Braz Oral Res*. 2011;25(1):80-84.
44. Kantrong N, Chang AM, Bamashmous S, Hajjar AM, Bucala RJ, Darveau RP. Macrophage migration inhibitory factor regulates specific innate immune sensor responses in gingival epithelial cells. *J Periodontol*. 2022;93(12):1940-1950.
45. Lin P, Niimi H, Ohsugi Y, et al. Application of ligature-induced periodontitis in mice to explore the molecular mechanism of periodontal disease. *Int J Mol Sci*. 2021;22(16):8900.
46. Hays A, Duan X, Zhu J, et al. Down-regulated Treg cells in exacerbated periodontal disease during pregnancy. *Int Immunopharmacol*. 2019;69:299-306.
47. González-Jaranay M, Téllez L, Roa-López A, Gómez-Moreno G, Moreu G. Periodontal status during pregnancy and postpartum. *PLoS One*. 2017;12(5):e0178234.
48. de Castro Bras LE, Frangogiannis NG. Extracellular matrix-derived peptides in tissue remodeling and fibrosis. *Matrix Biol*. 2020;91-92:176-187.
49. Paiva KBS, Granjeiro JM. Bone tissue remodeling and development: focus on matrix metalloproteinase functions. *Arch Biochem Biophys*. 2014;561:74-87.
50. Hernandez M, Valenzuela MA, Lopez-Otin C, et al. Matrix metalloproteinase-13 is highly expressed in destructive periodontal disease activity. *J Periodontol*. 2006;77(11):1863-1870.
51. Ejeil AL, Igondjo-Tchen S, Ghomrasseni S, Pellat B, Godeau G, Gogly B. Expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in healthy and diseased human gingiva. *J Periodontol*. 2003;74(2):188-195.
52. Franco C, Patricia HR, Timo S, Claudia B, Marcela H. Matrix Metalloproteinases as regulators of periodontal inflammation. *Int J Mol Sci*. 2017;18(2):440.
53. Otenio CC, Fonseca I, Martins MF, et al. Expression of IL-1β, IL-6, TNF-α, and iNOS in pregnant women with periodontal disease. *Genet Mol Res*. 2012;11(4):4468-4478.
54. Mesa F, Pozo E, O'Valle F, et al. Relationship between periodontal parameters and plasma cytokine profiles in pregnant woman with preterm birth or low birth weight. *Clin Oral Investig*. 2016;20(4):669-674.
55. Abusleme L, Moutsopoulos NM. IL-17: overview and role in oral immunity and microbiome. *Oral Dis*. 2017;23(7):854-865.
56. Di Stefano M, Polizzi A, Santonocito S, Romano A, Lombardi T, Isola G. Impact of oral microbiome in periodontal health and periodontitis: a critical review on prevention and treatment. *Int J Mol Sci*. 2022;23(9):5142.
57. Jensen J, Liljemark W, Bloomquist C. The effect of female sex hormones on subgingival plaque. *J Periodontol*. 1981;52(10):599-602.
58. Mealey BL, Moritz AJ. Hormonal influences: effects of diabetes mellitus and endogenous female sex steroid hormones on the periodontium. *Periodontol 2000*. 2000;2003(32):59-81.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Ortiz-Sánchez BJ, Juárez-Avelar I, Andrade-Meza A, et al. Periodontitis exacerbation during pregnancy in mice: Role of macrophage migration inhibitory factor as a key inductor. *J Periodont Res*. 2023;00:1-13. doi:10.1111/jre.13211

13 Anexo II Articulo Revisión

Hindawi
Mediators of Inflammation
Volume 2021, Article ID 5834608, 12 pages
https://doi.org/10.1155/2021/5834608

Review Article

Influence of Gestational Hormones on the Bacteria-Induced Cytokine Response in Periodontitis

Betsaida J. Ortiz-Sánchez,[1] **Martha Legorreta-Herrera**,[2] **and Miriam Rodriguez-Sosa**[3]

[1]*Dental Surgeon Career, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México (UNAM), MEX. C.P. 54090, Tlalnepantla, Mexico*
[2]*Molecular Immunology Laboratory, Facultad de Estudios Superiores, Zaragoza, UNAM, Iztapalapa, Mexico City C.P. 09230, Mexico*
[3]*Innate Immunity Laboratory, Facultad de Estudios Superiores Iztacala, UNAM, MEX C.P. 54090, Tlalnepantla, Mexico*

Correspondence should be addressed to Miriam Rodriguez-Sosa; rodriguezm@unam.mx

Received 27 July 2021; Revised 23 September 2021; Accepted 27 September 2021; Published 18 October 2021

Academic Editor: Gaetano Isola

Copyright © 2021 Betsaida J. Ortiz-Sánchez et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Periodontitis is an inflammatory disease that affects the supporting structures of teeth. The presence of a bacterial biofilm initiates a destructive inflammatory process orchestrated by various inflammatory mediators, most notably proinflammatory cytokines, which are upregulated in the gingival crevicular fluid, leading to the formation of periodontal pockets. This represents a well-characterized microbial change during the transition from periodontal health to periodontitis; interestingly, the gestational condition increases the risk and severity of periodontal disease. Although the influence of periodontitis on pregnancy has been extensively reviewed, the relationship between pregnancy and the development/evolution of periodontitis has been little studied compared to the effect of periodontitis on adverse pregnancy outcomes. This review is aimed at summarizing the findings on the pregnancy-proinflammatory cytokine relationship and discussing its possible involvement in the development of periodontitis. We address (1) an overview of periodontal disease, (2) the immune response and possible involvement of proinflammatory cytokines in the development of periodontitis, (3) how bone tissue remodelling takes place with an emphasis on the involvement of the inflammatory response and metalloproteinases during periodontitis, and (4) the influence of hormonal profile during pregnancy on the development of periodontitis. Finally, we believe this review may be helpful for designing immunotherapies based on the stage of pregnancy to control the severity and pathology of periodontal disease.

1. Introduction

Periodontal disease is an inflammatory condition of periodontal tissues with a heterogeneous aetiology and is one of the most common diseases in the world [1]. This disease affects the gum and supporting tissues of the teeth, alveolar bone, periodontal ligament, and root cementum. Approximately 60% of the total population has some degree of periodontal disease [2]. In Latin America, this number increases to up to 90% [3]. The early stage of development of this pathology is called gingivitis and affects only the soft tissues. The severe form, called periodontitis, severely affects periodontal tissues, mainly alveolar bone, with subsequent loss of insertion of dental organs.

The development of periodontal disease and its progression depend on different factors that modulate the host immune response against the biofilm, such as genetic and epigenetic predisposition including hereditary angyoderma [4], social factors, habits (such as tobacco and alcohol use and poor oral hygiene) [5], advanced age [6], and systemic conditions, such as obesity, malnutrition [7], infections (such as HIV/AIDS), osteoporosis and stress [8], type 1 and 2 diabetes [9], and scleroderma disease [10]. Notably, periodontitis affects 23% of women between 23 and 54 years of age and is present in 56% of pregnant women [11]. Furthermore, recent evidence suggests that hormonal treatment, the use of hormonal contraceptives, and pregnancy induce clinical, cytological, or microbiological changes in women

[12], which probably promote the development of this disease.

Inflammatory cytokines are upregulated during pregnancy and increased during ovulation, in early gestation, in term pregnancy, and during delivery [13]. However, it has not been established whether there is any relationship between pregnancy/proinflammatory cytokines and the development of periodontal disease. In this review, we summarize the current knowledge by providing a broad overview of periodontitis and then focusing specifically on recent findings related to the inflammatory response in pregnancy and its possible relationship to the development of periodontitis.

2. Periodontitis and the Oral Microbiome

The oral cavity has a dynamic environment that is formed by the oral microbiome with all of its interspecies interactions but also interactions with the oral cavity, creating a symbiotic relationship with the human host [14]. Periodontitis is initiated by polymicrobial synergy, and dysbiosis is modified by numerous risk factors. Competitive and cooperative interspecies interactions of microbial communities can shape the nature and function of the entire microbiome synergism [15]. The subgingival microbiome includes the red complex triad (*Treponema denticola*, *Tannarella Forsythia*, and *Porphyromonas gingivalis*) [16], orange complex triad (*Fusobacterium nucleatum*, *Prevotella intermedia*, and *Parvimonas micra*), *Actinobacillus actinomycetemcomitans*, *Campylobacter rectus*, *Eikenella corrodens*, *Bacteroides forsythus* [17], *Filifactor alocis* [18], *Peptoanaerobacter stomatis*, *Firmicutes phylum*, *Methanobrevibacter oralis* [19], *C. albicans* [20], and *human cytomegalovirus* and *Epstein-Barr virus* [21].

The periodontal microbiome is complex and constitutes the cornerstone in the development of periodontal disease. The characteristics of the bacteria themselves are essential in determining the course of the immune response. For example, *Porphyromonas gingivalis* can modulate the innate inflammatory response [22]. *Filifactor alocis* also induces oxidative stress and alters the recognition capacity of the inflammatory response by inactivating the complement pathways [23]. *Filifactor alocis* and other bacteria, such as *Porphyromonas gingivalis*, are highly invasive and promote dysbiosis of the microbiota [24]; therefore, a pathogenesis model has been proposed in which periodontal disease is initiated due to dysbiosis of the microbiota, called the PSD (polymicrobial synergy and dysbiosis) model [25].

3. Immune Response in Periodontitis

Immune cells interact with biofilms when their pattern recognition receptors (PRRs) detect pathogen-associated molecular patterns (PAMPs) present on bacteria. These receptors are expressed on innate immune cells, such as neutrophils, eosinophils, basophils, macrophages (Mφs), monocytes, dendritic cells (DCs), and natural killer (NK) cells, and adaptive immune cells, such as T and B lymphocytes, as well as on nonimmune cells, such as epithelial cells, endothelial cells, and fibroblasts [26]. This PAMP-PPR interaction activates the innate immune response characterized by neutrophil, eosinophil, and basophil recruitment, consequently activating the complement system [27].

This first recognition is characterized by acute inflammation; if biofilm dysbiosis persists, this response develops into chronic inflammation. In this phase, osteoclast activation is favoured. It results in bone resorption, with subsequent degradation of the bone matrix and periodontal ligament fibres by metalloproteinases (MMPs) and the formation of granulation tissue [28] (Figure 1). Thus, in both acute inflammation and chronic inflammation, cytokines, and inflammatory mediator's determinate disease progression factors.

Antigen-presenting cells (APCs), such as DCs, recognize pathogens expressing PAMPs, internalize these pathogens by phagocytosis, and degrade and process pathogen-derived antigens, transforming the antigens into small peptides that bind to major histocompatibility complex (HLA) molecules for display on the cell surface. This presentation is accompanied by the expression of the costimulatory molecules CD86 and CD40. DCs migrate to secondary lymphoid tissues (lymph nodes and lymphoid tissue) to present antigens and thus activate $CD4^+$ T cells to generate an antigen-specific immune response [29]. CD4+ T cells differentiate into regulatory and effector T cell subsets: Th1, Th2, Th17, follicular helper T (Tfh) cells, and regulatory T cells (Tregs) [30]. The differentiation of Th1 and Th2 cells is mutually antagonistic; Treg and Th17 cells share the same origin and have opposite effects, while Th17 cells cause autoimmunity and inflammation, and Treg cells inhibit these and maintain immune homeostasis.

The activation profile of $CD4^+$ T lymphocytes in periodontal disease varies depending on disease progression. In the initial phase, $CD4^+$ T lymphocytes exhibit a proinflammatory Th-1 profile characterized by the synthesis of macrophage inhibitory factor (MIF), interleukin- (IL-) 2, and interferon- (IFN-) γ, which promote cellular immunity and the activation of cytotoxic $CD8^+$ T lymphocytes (TCs) and Th-17 cells [31]. Other cytokines, such as IL-1α, IL-1β, IL-8, IL-6, and tumour necrosis factor- (TNF-) α produced by monocytes, Mφs, DCs, and neutrophils, are also produced under these conditions [32]. In addition, endothelial cells, fibroblasts, and osteoclasts produce prostaglandin E2 (PGE2) and granulocyte macrophage colony-stimulating factor (GM-CSF) [33]. Together, these conditions promote the expression of receptor activator of NF-κB ligand (RANKL), leading to osteoclastogenesis [34].

In the chronic phase of periodontitis, $CD4^+$ T lymphocytes differentiate towards an anti-inflammatory Th2 profile, characterized by the production of IL-4, IL-5, IL-6, and IL-10 [35]. This profile favours B lymphocyte activation and subsequent differentiation into IgG-type immunoglobulin-producing plasma cells [36]. In this way, when periodontitis becomes chronic, negative regulation of inflammation through the anti-inflammatory cytokines IL-4, IL-6, IL-10, IL-11, and IL-13 becomes predominant [37]. This immunoregulation is a complex phenomenon involving mediators such as RANKL-DCs, favouring the activation of $CD4^+$

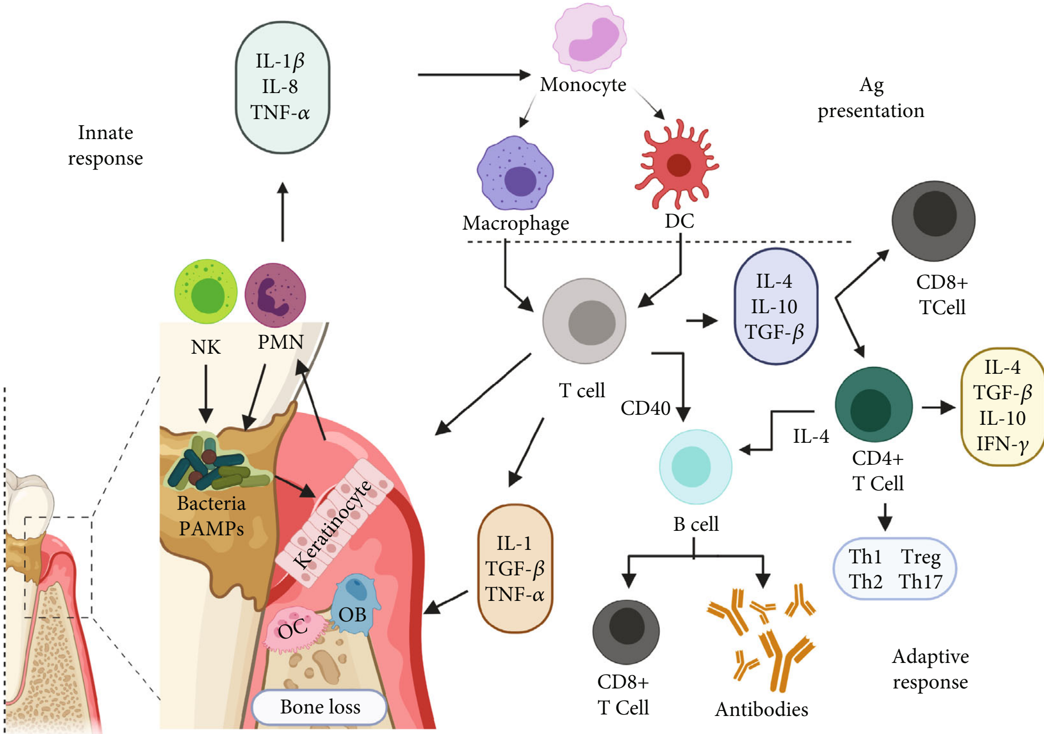


Figure 1: Innate immune cells, including keratinocytes, polymorphonuclear cells (PMNs), antigen-presenting cells (APCs), and natural killer (NK) cells, hold pathogen recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) present in biofilm bacteria, and these interactions promote the acute inflammatory response. Antigen-presenting cells (APCs) phagocytose pathogens, process antigens, and present the antigens in the form of peptides displayed by major histocompatibility complex (HLA) molecules. Costimulatory molecules stabilize this interaction. APCs migrate to secondary lymphoid tissues where they activate adaptive immune cells, including CD4$^+$ T cells, such as Th1, Th2, and Th17 cells; cytotoxic CD8$^+$ T cells; and B cells that will mature into antibody-producing plasma cells. Created with BioRender.com (https://biorender.com/).

Foxp3$^+$ T (Treg) cells [38], which also regulate the inflammatory Th1 response, thus, preventing the destruction of periodontal tissues [39].

Th17 cells are characterized by the production of the cytokine IL-17, although they also produce other cytokines, such as IL-17F, IL-21, IL-22, and GM-CSF [40]. Th17 cells are induced in the presence of TGF-β/IL-1β, IL-6, and IL-23 and express chemokine receptor- (CCR-) 6, which allows their migration to barrier and mucosal sites, such as gingival tissues, suggesting a protective role in the oral barrier. The inflammatory functions of Th17 cells depend on the different combinations of cytokines expressed in the local environment [33].

IL-17 signalling on epithelial cells is essential for the physiological regulation of mucosal immunity and barrier defences, promotes the production of antimicrobial factors, regulates the recruitment and generation of neutrophils through the induction of chemokines CXCL-1, 2, and 5, and induces the secretion of granulopoietic factors such as G-CSF and GM-CSF. Moreover, IL-17 induces the production of antimicrobial mediators such as β-defensins (HBD), regenerative proteins (ReG), S100 proteins, cathelicidins, lipocalins, and lactoferrins [40].

In periodontal lesions, an increase in Th17-related cytokines, such as IL-23 and IL-21, and other proinflammatory and osteoclastogenic mediators, such as IL-6 and RANKL, has been found [41]. IL-17 can enhance RANKL expression on osteoblasts by promoting the secretion of MMP-1, MMP-3, IL-6, and IL-8 from gingival fibroblasts and TNF release from macrophages in periodontal tissues [42] and activate RANK signalling on osteoclasts, promoting osteoclastogenesis [43]. In addition, IL-17 enhances inflammation through excessive neutrophil recruitment, enhances proinflammatory cytokine production, and activates osteoclasts, contributing to immunopathology and bone destruction.

In addition, the presence of Treg cells inhibits osteoclast formation and monocyte/Mφ differentiation through the secretion of transforming growth factor- (TGF-) β, IL-4, and IL-10 and the interaction of CTLA-4 (cytotoxic T-lymphocyte antigen) with the monocyte precursor/Mφ receptors CD80/CD86 [44].

Keratinocytes express several families of pattern recognition receptors, including TLR2, TLR4, NOD1, and NOD2, which are activated by both extracellular and intracellular bacterial molecular structures.

The mechanisms of tolerance include not only DCs but also Tregs. The function of oral Langerhans cells (LCs) under physiological conditions is to maintain a state of immune tolerance [45]. DCs also participate in peripheral tolerance in chronic periodontitis. These cells are capable of phagocytosing pathogens, but due to the anti-inflammatory cytokines IL-10 and TGF-β, their ability to

present antigens decreases; this decrease is associated with a deficiency in the costimulatory molecules CD80 and CD86, so they cannot activate T cells properly [29]. Natural killer cells, either through direct cell-to-cell contact or indirectly through cytokines, interact with dendritic cells to mediate T cell immune responses [46].

In summary, the severity of the pathology of periodontal disease, as well as its chronicity, depends on the balance and interaction between the Th1/Th17 inflammatory response and the Th2/Treg anti-inflammatory regulatory response [47]. The Treg/Th17 balance is shifted in favour of Th17 cells in the presence of proinflammatory cytokines.

4. Remodelling of Bone Tissue in Periodontitis

Bone tissue is one of the most affected tissues in periodontitis; under normal conditions, it is constantly remodelled, which requires cells that degrade the bone matrix (osteoclasts) and cells that synthesize the bone matrix (osteoblasts) [48]. Briefly, the bone matrix produces the growth factors TGF-β and insulin-like growth factor- (IGF-) 1. Both molecules favour the recruitment of preosteoblasts and promote their maturation; subsequently, some osteoblasts differentiate into osteocytes. This mechanism is regulated by paracrine and endocrine factors, such as epinephrine B2, IL-6, and parathyroid hormone (PTH) [49].

On the other hand, osteoclasts differentiate from a myeloid precursor under the influence of Mφ-colony-stimulating growth factor (M-CSF) and RANKL. Osteoprotegerin (OPG), produced by osteoblasts, modulates the osteoclast differentiation process [50]. Osteoclasts produce the proteolytic enzymes cathepsin K and metalloproteinases (MMPs), which degrade the bone matrix. In addition, H^+ proton transporters and ATPase generate an acidic environment that, together with chloride channels, hydrolyses, and solubilizes both organic matter and inorganic matter. All this happens in Howship's lacunae, and the osteoclasts seal them with their podosomes [51].

In periodontitis, lymphocyte infiltrates and mononuclear cells influence and alter the homeostatic balance of the bone. Although modulation of the bone immune system is complex, the balance of proinflammatory Th1 and anti-inflammatory Th2 immune responses is critical [52]. The cytokines that promote bone resorption include IL-1β, TNF-α, IL-6, IL-15, and IL-17, and the cytokines that inhibit bone resorption include IL-4, IL-10, IL-13, IL-18, GM-CSF, and IFN-γ [50]. The best example is TNF-α, which activates osteoclasts and inhibits osteoblast differentiation with a consequent decrease in bone formation [53]. Specifically, TRAIL (TNF-related apoptosis-inducing ligand) participates in osteoblast apoptosis and low bone quality in periodontitis [54].

The other important factor in bone resorption is a member of the TNF family, RANKL, which promotes osteoclast differentiation and modifies the relationship between osteoblasts and osteoclasts [55]. RANKL is overexpressed in proinflammatory systems, and the major source is B lymphocytes, followed by T lymphocytes and finally monocytes, although osteoblasts also produce RANKL after activation through TLRs [56].

The RANKL-RANK-OPG system is involved in bone regulation via regulation of the immune system to control other systems and several pathologies. These interactions have been described mainly in rheumatoid arthritis, which involves bone loss and bone remodelling [57, 58]. The regulation of the RANKL-RANK-OPG system and its mechanisms should be clarified in periodontitis since modulation of these mechanisms may favour treatment and prevent disease sequelae.

4.1. Involvement of Metalloproteinases in Bone Remodelling. Periodontal tissues are composed of connective tissue; the extracellular matrix (ECM) is mainly formed by collagen types I, III, IV, V, and VI and noncollagenous proteins, including elastin, fibronectin, laminins, and proteoglycans. In periodontitis, significant degradation of all of these constituent elements of periodontal tissues occurs. Overexpression of MMPs, a family of zinc-dependent endopeptidases, is associated with the development and severity of periodontal structure loss in this pathology. These enzymes are capable of degrading most of the components of the ECM [59]. In addition, MMPs favour processes involved in inflammation, such as inflammatory cell migration, chemokine recruitment and processing, cleavage and neutralization of complement components, phagocytosis, and cell lysis [60].

Different MMPs have the specificity to act in the degradation of specific types of tissues, e.g., MMP-2 in its proenzyme form (~72 kDa) and in its active form (~59–62 kDa) and the MMP-9 proenzyme (~92 kDa) and active forms (~88 kDa) degrade fibronectin, elastin, and collagen types IV, V, VII, X, XI, and XII; in acidic medium, they can degrade collagen type I [61]. MMP-13, both in its proenzyme form (~60 kDa) and in its active form (~45–50 kDa), degrades collagen I, collagen II, collagen III, collagen IV basal membrane, proteoglycans, fibronectin, fibrin, and tenasin [62].

The mechanisms that regulate homeostasis, such as the overexpression of MMPs in periodontitis, are complex processes. In homeostasis, one of the regulatory pathways involves tissue inhibitors of MMPs (TIMPS) and α2-macroglobulins that bind covalently and irreversibly to the active site of MMPs with high affinity. The levels of these endogenous mediators are elevated in healthy tissue and various fluids, such as serum, amniotic fluid, and saliva, and these mediators are synthesized by fibroblasts, monocytes, Mφs, endothelial cells, and osteoblasts [63]. Another mechanism of negative regulation of MMP expression is the presence of the oestrogen 17β-oestradiol, which negatively regulates the flow of calcium into cells [64] and consequently reduces the expression of MMPs, particularly MMP-1 [65].

On the other hand, the overexpression of MMPs can be triggered by different factors, such as the presence of PGE_2 [66], *in vitro* and *in vivo* are influenced by mechanical load as orthodontic movement [67, 68], interactions with periodontopathogenic bacteria, such as *Eikenella corrodens* [69], *Porphyromonas gingivalis*, and *Prevotella intermedia* [70], or polysaccharides and cytokines, such as IL-1β and TNF-

Table 1: Influence of sex hormones on some cells and cytokines of the immune response.

Hormone	Regulation	References
17β-estradiol	↑TCD8+ from spleen and in vitro.	[75]
	↑ maturation and activation of B lymphocytes, ↓Ig2a in peripheral blood mononuclear cells and spleen cells.	[76, 77]
	↓TNF-α, ↑IFN-γ e ↑IL-10 in peripheral blood mononuclear cells, spleen, and in vitro.	[78, 79]
	Peritoneal Mφ ↑, TLR4 in vitro.	[80]
	↑DCs ↑IL-12 in bone marrow, in vivo.	[81]
	Inhibits apoptosis by TNF-α via PI3k/Akt in neural progenitor cells.	[82]
	↓IL-1β and TNF-α in bone marrow.	[83]
	[↓E2] ↑Th1, [↑E2] ↑Th2 in peripheral blood mononuclear cells in vitro.	[84]
Progesterone	↓Mφ, DCs, and NKs in peripheral blood mononuclear cells	[85]
	↓NFκB transduction.	[86]
	↑Th2, ↑IL-4 e ↑IL-5, ↑Tregs, and ↓TH17 in peripheral blood mononuclear cells.	[87, 88]
Testosterona	↓LB, ↑apoptosis in bone marrow and lymph nodes.	[89]
	↑TCD8+ in peripheral blood mononuclear cells.	[90]
	↑Mφ, ↑TNF-α, ↑CCR2, ↑[IL-10], and ↓IFN-γ in skin and spleen.	[91, 92]
	Mφ, ↑IL-12 e ↑IL-1β in vitro.	[93]
	DCs ↓TNF-α, nitric oxide, TLR-4.	[94, 95]
	↓IgG e IgM, peripheral blood mononuclear cells.	[96]
	↑TGF-β e ↑IGFs ↑bone apposition, ↓ IL-6 osteoclastogenesis.	[97, 98]

α. In any case, these factors act on monocytes and Mφs by favouring the production of mediators that function as activators or modulators of MMPs [71]. For example, MMP-13 upregulates RANKL/OPG levels by activating MMP-9, increases TGF-β signalling in metastatic bone lesions [72], and influences osteoclastic activity [73].

Undoubtedly, the participation of MMPs in the development and severity of periodontitis is known. Establishing whether their expression is affected by hormonal conditions, such as gestation, is important because regulating their expression could be considered a component of the therapeutic treatment of gestational periodontitis.

5. Sex Hormones and Periodontitis

Sex hormones modulate immune functions, such as thymocyte maturation and selection, cell migration, MHC-II expression, cell proliferation, and cytokine production (Table 1) [74].

The regulatory effects exerted by hormones on the immune response depend on interactions with their receptors. For example, B cells have high expression of the genes encoding the two oestrogen receptors, ER1 and ER2. There is a moderate expression of these receptors on $CD4^+$ T, $CD8^+$ T, NK, and plasmacytoid DCs, while monocytes express reduced levels of ER1. Estradiol and ERs bind to transcription factors, such as NFκB, SP1, AP-1, and C/EBPβ, that are involved in the regulation of different cellular functions [99].

Progesterone receptors (PRs) are present on epithelial cells, mast cells, eosinophils, NK cells, Mφs, plasmacytoid DCs, and $CD4^+$ and $CD8^+$ T lymphocytes. Interestingly, the expression of PRs is higher in DCs from female rats than in those from male rats [100], which makes it clear that the expression of these receptors and consequently the response that is generated when their ligand binds are higher in females. Different PRs include two intracellular receptors (iPRs) and three membrane receptors (mPRs), with two isoforms each. iPRs were initially described in the lymphocytes of pregnant women, while mPRs were described in T lymphocytes and are overexpressed during the luteal phase in $CD8^+$ T lymphocytes. Differential expression of PRs may partially explain the differential activation of immune cells and differences in susceptibility to various infectious and noninfectious diseases between men and women [101].

In the same context, the sex hormone profile also has an impact on subgingival microbiology. It has been demonstrated that this profile promotes the development of periodontopathogenic bacteria, such as *Porphyromonas gingivalis* [102], subgingival anaerobic-aerobic bacteria, *Prevotella melaninogenica*, and *Prevotella intermedia* [103]. It is widely recognized that hormones related to gestation alter the immune response, modifying the pathogenesis of some diseases; for example, in multiple sclerosis and autoimmune encephalomyelitis, where an exacerbated inflammatory response is associated with the severity of the pathology, the disease severity decreases during gestation. Diseases such as malaria and influenza, which require acute inflammatory responses for their control, are exacerbated during pregnancy [80]. This phenomenon could be associated with estriol concentrations, which increase significantly during gestation. Estriol is produced in high concentrations by the fetoplacental unit during pregnancy; it accounts for 90% of the oestrogen produced during pregnancy, while the other 10% corresponds to oestradiol [104]. Although the immunological functions of estriol are similar to those of oestradiol because they share receptors, estriol seems to differentially influence the immune response; in experimental models of autoimmune pathologies, when estriol was administered, decreases in the proinflammatory cytokines TNF-α and IFN-γ have been observed, in addition to decreases in $CD4^+$ and $CD8^+$ cells [105]. This immune response modified

by the presence of estriol, together with other hormones present during pregnancy, could also influence the development of periodontitis. This observation is corroborated in pregnant women, who, due to their condition, have a modified hormonal profile that consequently favours the accumulation of *Bacteroides*, which is increased in abundance up to 55 times in pregnant women compared to nonpregnant women [106].

The involvement of hormones other than estriol in the development of periodontal diseases has been widely documented. Progesterone increases vascular permeability and favours oedema, erythema, and gingival bleeding, which are all associated with increased populations of *Porphyromonas gingivalis*, *Prevotella intermedia* [107], *Actinobacillus actinomycetemcomitans* [108], and *Prevotella melaninogenica* [109].

Oestrogens, particularly oestradiol, favour angiogenesis and fibroblast proliferation and promote osteoblast differentiation and maturation, osteoprotegerin (OPG) and RANKL expression in osteoblasts, and osteoclast apoptosis by inhibiting osteoclast activity [83]. Periodontal ligament (PLD) cells synthesize RANKL and OPG. *In vitro* cultures of oestrogen-treated PLD cells increase OPG expression and decrease RANKL expression through ER2 [110]; these observations demonstrate that oestrogens can modulate the activity of periodontal tissues and promote homeostasis.

Androgens participate in bone growth; they are anabolic agents that increase bone mass, mainly in males, although different androgens, including testosterone, are also present in females. Androgen receptor mRNA is expressed more in cortical osteoblasts than in trabecular bone and is more closely related to cortical osteoblasts, which generate a thicker cortical bone layer in males, while in osteoblasts, androgen receptor mRNA is expressed similarly between the sexes. Androgens promote osteoblast differentiation and decrease osteoclast apoptosis; specifically, dihydrotestosterone reduces OPG levels. The functions of androgens in women have not been clearly defined; however, they are involved in the maintenance of bone density [100].

Sex hormones are involved in bone regulation and immune system maturation and modulate the function of nonsexual tissues; therefore, these hormones may play a central role in the development of periodontitis in different stages of life.

5.1. Pregnancy and Periodontitis. Gestation is a condition that involves physiological changes in the mother, and these changes should allow "immune tolerance" towards the foetus to develop, as well as the appearance of new cells, such as trophoblasts [111].

Recently, the relevance of the model of a foetus as a semiallograft capable of inducing the absence of a specific immune response to prevent its destruction has been debated. It is not a simple absence of the immune response but a state of immunoregulation that allows the implantation of a foetus, which is also able to respond to injury or aggression from the environment with an immune response endowed with specificity and memory [112]. This immune tolerance, in order to not reject the foetus and at the same time allow protection of pregnant woman against pathogens, requires the transient modification of immunity, which favours a Th2 environment over a Th1 environment [113]. Different mechanisms have been described to explain immune tolerance to paternal antigens, including tolerance induction in T lymphocytes, including Treg and Th17 cells [114]. In a healthy pregnancy, the Th17/Treg ratio shifts in favour of Treg cells, while a decrease in Treg cells or an increase in Th17 cells is detrimental to normal pregnancy [115]. Tolerance is promoted by Treg and Th2 cells by repressing Th1 and Th17 cells, while Th17 cells protect trophoblasts from pathogens [116].

During gestation, the maternal-foetal interaction and the development of the placenta favour increased hormone concentrations. In particular, the placenta synthesizes and releases oestrogen and progesterone into circulation. This initiates events that stimulate "suppressive" immune responses, mainly at the level of lymphocytes. Suppression of $CD4^+$ and $CD8^+$ T lymphocytes decreases the secretion of IL-2, IFN-γ, TNF-β, TNF-α, IL-1β, and IL-6 [117]. The levels of oestradiol in human serum are ~0.1 μM, and those in blood from the intervillous space are ~0.25 μM, which is ~25 times higher than the concentration found in nonpregnant women at the midovarian cycle stage [118]. Oestradiol at a concentration of 0.04 ng/mL or higher and progesterone at 0.1 ng/mL both inhibit lipopolysaccharide- (LPS-) induced IL-1 and TNF-α secretion in monocyte cultures. In addition, the switch from the Th1 profile to the Th2 profile and the suppression of the cytolytic function of NK cells are processes regulated by progesterone-induced blocking factor (PIBF), which is secreted by $CD8^+\gamma\delta$ T cells [119]. High concentrations of PIBF favour the differentiation of $CD4^+$ T cells into Th2 lymphocytes, which increases IL-4, IL-5, and IL-10 concentrations and promotes the prevalence of an anti-inflammatory profile (Figure 2) [120].

In the gestational stage, polymorphonuclear cells (PMNs) show decreased chemotaxis and adhesion beginning in the second trimester and continuing throughout gestation [121]. This altered neutrophil activation and depressed leukocyte function during pregnancy may explain susceptibility to certain infections [122]. For example, gingival inflammation has been associated with increased serum levels of oestrogen and progesterone, even though no changes in TNF-α or IL-1β levels have been detected [123].

In animal models, levels of the cytokines IL-1β, IL-6, IL-8, IL-17, and TNF-α increase under different conditions. For example, in maternal infections with periodontal pathogens or *in vitro* models of placental cells and tissues, exposure to periodontal bacteria or products induces the secretion of COX-2, IL-8, IFN-γ, and TNF-α in addition to causing apoptosis [124], and PGE_2 causes uterine contractions [125].

On the other hand, during pregnancy, periodontal alterations increase, and the ratio of anaerobic to aerobic bacteria is modified in the second trimester of pregnancy, mainly through increases in *Prevotella melaninogenica* and *Prevotella intermedia* related to the plasma concentrations of estrogens and progesterone [126]. In animal models of periodontitis established with *Porphyromonas gingivalis* in pregnant mice, an increased immune response with decreased expression of anti-inflammatory cytokines and increased

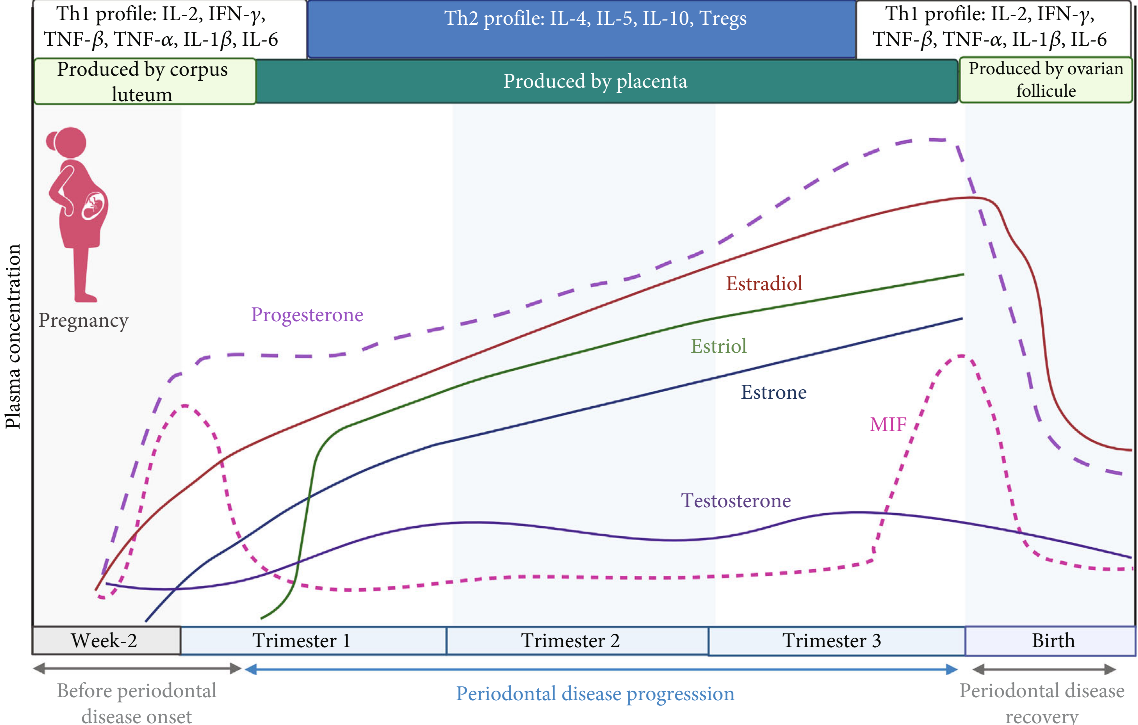


Figure 2: Time course of pregnancy hormones, periodontal disease, and the proinflammatory cytokine MIF. During early gestation, hormones are produced in the corpus luteum, and later until the end of gestation, they are produced in the placenta. Estriol is also synthesized in the placenta, and its production ceases when the pregnancy reaches term. Therefore, embryo implantation and early gestation require a Th1 profile with the expression of proinflammatory cytokines. During the second half of gestation, the expression of these cytokines decreases, and the profile is a Th2 and Treg cell profile, which is maintained until delivery, when the profile returns to a Th1 profile. The expression of MIF, which is a proinflammatory cytokine, maintains this trend during pregnancy. Created with BioRender.com (https://biorender.com/).

destruction of periodontal tissues is observed [102], and an imbalance in the Th17/Treg cell ratio with aggravation of periodontitis during pregnancy occurs [127].

Periodontitis causes gynecological problems, ranging from difficulty in embryo implantation to preterm delivery and low birth weight. Two possible causes have been proposed: first, periodontal bacteria cause infections in the placenta and foetus; second, inflammation can provoke responses at the maternal-foetal interface [128]. There are several contrasting and inconclusive reports on patients with recurrent miscarriages and multiple implantation failures during *in vitro* fertilization cycles which have a prevalent Th1 profile in their peripheral blood lymphocytes [129].

Offenbacher et al. noted that primary infections in distant systems can guide a pregnancy to an abnormal term [130]. Periodontal disease is an infectious process in periodontal tissues characterized by an increase in proinflammatory cytokines, and during pregnancy, the concentration of prostaglandins increases [35]. Therefore, there may be a relationship between both factors; periodontitis influences pregnancy, and that gestation influences the severity of periodontitis. One of the possible causes is the spread of bacteria or inflammatory mediators of periodontal origin by different routes, including (1) bacterial blood spread (bacteremia), (2) blood dissemination of inflammatory mediators, and (3) transmission of oral pathogens and colonization of the vaginal microbiome [131]. González-Jaranay et al. reported that in pregnant women with some degree of periodontitis, symptoms progress and worsen throughout gestation. However, in the postpartum period, clinical data improve [132]. Other authors have noted that maternal periodontal disease is not a risk factor if infectious processes are controlled [133].

Regarding the interaction of gestation with periodontitis, some studies did not find strong evidence of this interaction; however, they proposed routine periodontal therapy in pregnant women as a safe treatment for mothers and foetuses, in addition to improving the clinical signs of maternal periodontal disease [134]. In contrast, evidence of strong links between periodontitis and pregnancy disorders such as preeclampsia, preterm delivery, and low birth weight, attributable to periodontal disease, has recently been reported [135], as have associations of periodontitis with metabolic disorders such as obesity and diabetes [136].

6. Conclusions

This review shows that sex hormones modulate the immune response and participate in processes such as the maturation and selection of immune cells, cell trafficking, expression of histocompatibility molecules, cell proliferation, and cytokine production. Although pregnancy is a condition that modifies

the hormonal profile, little is known about its effects on the development of periodontitis. Here, we collect important evidence that gestational hormones, such as 17β oestradiol, estriol, and progesterone, influence the development of periodontitis. Importantly, the interaction between the concentration of gestational hormones and periodontal disease appears to be bidirectional: on the one hand, the hormonal profile during pregnancy seems to be decisive for the development and severity of periodontal disease, but on the other hand, the infectious process associated with periodontitis during pregnancy generates a proinflammatory immune profile that can produce alterations such as preeclampsia, preterm delivery, and low birth weight.

However, future studies are needed to understand the immune mechanisms underlying the interaction of pregnancy and periodontal diseases. The information gathered here has the potential to contribute to an understanding of the role of hormones in the development of periodontitis, allowing dental teams that care for pregnant and childbearing women to develop preventive and therapeutic strategies.

Data Availability

No data were used to support this study.

Conflicts of Interest

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

Acknowledgments

Betsaida Julieta Ortiz-Sánchez is a doctoral student from the Programa de Doctorado en Ciencias Biológicas, Universidad Nacional Autónoma de México (UNAM), and received a fellowship from the Council for Science and Technology of the State of Mexico (COMECyT; 2019-2020). This work was funded in part by the Support Program for Research Projects and Technological Innovation (PAPIIT)-UNAM, grant number (IN-217021) and by the National Council of Science and Technology of Mexico (CONACYT), grant number (A1-S-10463).

References

[1] H. C. Slavkin, "Does the mouth put the heart at risk?," *Journal of the American Dental Association (1939)*, vol. 130, no. 1, pp. 109–113, 1999.

[2] M. S. Tonetti, S. Jepsen, L. Jin, and J. Otomo-Corgel, "Impact of the global burden of periodontal diseases on health, nutrition and wellbeing of mankind: a call for global action," *Journal of Clinical Periodontology*, vol. 44, no. 5, pp. 456–462, 2017.

[3] A. Duque, "Chronic periodontitis in Latin America," *Revista clínica de periodoncia, implantología y rehabilitación oral*, vol. 9, no. 2, pp. 208–215, 2016.

[4] L. Ramaglia, G. Isola, G. Matarese et al., "Prophylaxis of acute attacks with a novel short-term protocol in hereditary angioedema patients requiring periodontal treatment," *Oral Health & Preventive Dentistry*, vol. 18, no. 1, pp. 355–361, 2020.

[5] C. Verrusio, V. Iorio-Siciliano, A. Blasi, S. Leuci, D. Adamo, and M. Nicolò, Eds., "The effect of orthodontic treatment on periodontal tissue inflammation: a systematic review," *Quintessence International*, vol. 49, no. 1, pp. 69–77, 2018.

[6] Y. C. Liu, U. H. Lerner, and Y. T. Teng, "Cytokine responses against periodontal infection: protective and destructive roles," *Periodontology 2000*, vol. 52, no. 1, pp. 163–206, 2010.

[7] R. J. Genco, "Current view of risk factors for periodontal diseases," *Journal of Periodontology*, vol. 67, no. 10, pp. 1041–1049, 1996.

[8] C. O. Enwonwu and N. Salako, "The periodontal disease-systemic health-infectious disease axis in developing countries," *Periodontology 2000*, vol. 60, no. 1, pp. 64–77, 2012.

[9] G. Isola, G. Matarese, L. Ramaglia, E. Pedullà, E. Rapisarda, and V. Iorio-Siciliano, "Association between periodontitis and glycosylated haemoglobin before diabetes onset: a cross-sectional study," *Clinical Oral Investigations*, vol. 24, no. 8, pp. 2799–2808, 2020.

[10] G. Isola, R. C. Williams, A. Lo Gullo et al., "Risk association between scleroderma disease characteristics, periodontitis, and tooth loss," *Clinical Rheumatology*, vol. 36, no. 12, pp. 2733–2741, 2017.

[11] D. A. Webb, L. Mathew, and J. F. Culhane, "Lessons learned from the Philadelphia collaborative preterm prevention project: the prevalence of risk factors and program participation rates among women in the intervention group," *BMC Pregnancy and Childbirth*, vol. 14, no. 1, p. 368, 2014.

[12] B. L. Mealey and A. J. Moritz, "Hormonal influences: effects of diabetes mellitus and endogenous female sex steroid hormones on the periodontium," *Periodontology 2000*, vol. 32, no. 1, pp. 59–81, 2003.

[13] Y. Shimaoka, Y. Hidaka, H. Tada et al., "Changes in cytokine production during and after normal pregnancy," *American Journal of Reproductive Immunology*, vol. 44, no. 3, pp. 143–147, 2000.

[14] A. D. Proal, I. A. Lindseth, and T. G. Marshall, "Microbe-microbe and host-microbe interactions drive microbiome dysbiosis and inflammatory processes," *Discovery Medicine*, vol. 23, no. 124, pp. 51–60, 2017.

[15] A. Radaic and Y. L. Kapila, "The oralome and its dysbiosis: new insights into oral microbiome-host interactions," *Computational and Structural Biotechnology Journal*, vol. 19, pp. 1335–1360, 2021.

[16] R. J. Lamont, H. Koo, and G. Hajishengallis, "The oral microbiota: dynamic communities and host interactions," *Nature Reviews Microbiology*, vol. 16, no. 12, pp. 745–759, 2018.

[17] L. Tonoyan, S. Vincent-Bugnas, C. V. Olivieri, and A. Doglio, "New Viral Facets in Oral Diseases: The EBV Paradox," *International Journal of Molecular Sciences*, vol. 20, no. 23, p. 5861, 2019.

[18] B. J. Paster, S. K. Boches, J. L. Galvin et al., "Bacterial diversity in human subgingival plaque," *Journal of Bacteriology*, vol. 183, no. 12, pp. 3770–3783, 2001.

[19] H. T. Huynh, M. Pignoly, V. D. Nkamga, M. Drancourt, and G. Aboudharam, "The repertoire of archaea cultivated from severe periodontitis," *PLoS One*, vol. 10, no. 4, article e0121565, 2015.

[20] C. J. Nobile and A. D. Johnson, "Candida albicansBiofilms and human disease," *Annual Review of Microbiology*, vol. 69, no. 1, pp. 71–92, 2015.

[21] J. Slots, "Periodontal herpesviruses: prevalence, pathogenicity, systemic risk," *Periodontol 2000*, vol. 69, no. 1, pp. 28–45, 2015.

[22] F. E. Dewhirst, T. Chen, J. Izard et al., "The human oral microbiome," *Journal of Bacteriology*, vol. 192, no. 19, pp. 5002–5017, 2010.

[23] H. Y. Kim, Y. Lim, S. J. An, and B. K. Choi, "Characterization and immunostimulatory activity of extracellular vesicles fromFilifactor alocis," *Molecular Oral Microbiology*, vol. 35, no. 1, pp. 1–9, 2020.

[24] A. W. Aruni, K. Zhang, Y. Dou, and H. Fletcher, "Proteome analysis of coinfection of epithelial cells with Filifactor alocis and Porphyromonas gingivalis shows modulation of pathogen and host regulatory pathways," *Infection and Immunity*, vol. 82, no. 8, pp. 3261–3274, 2014.

[25] G. Hajishengallis and R. J. Lamont, "Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology," *Molecular Oral Microbiology*, vol. 27, no. 6, pp. 409–419, 2012.

[26] Y. Delneste, C. Beauvillain, and P. Jeannin, "Immunité naturelle," *Medical Science (Paris)*, vol. 23, no. 1, pp. 67–74, 2007.

[27] S. A. Khan, E. F. Kong, T. F. Meiller, and M. A. Jabra-Rizk, "Periodontal diseases: bug Induced, Host Promoted," *PLOS Pathogens*, vol. 11, no. 7, article e1004952, 2015.

[28] D. F. Kinane, P. G. Stathopoulou, and P. N. Papapanou, "Periodontal diseases," *Nature Reviews Disease Primers*, vol. 3, no. 1, 2017.

[29] M. Azuma, "Fundamental mechanisms of host immune responses to infection," *Journal of Periodontal Research*, vol. 41, no. 5, pp. 361–373, 2006.

[30] K. Hirahara and T. Nakayama, "CD4+ T-cell subsets in inflammatory diseases: beyond the Th1/Th2 paradigm," *International Immunology*, vol. 28, no. 4, pp. 163–171, 2016.

[31] F. Ietta, E. A. V. Ferro, E. Bevilacqua, L. Benincasa, E. Maioli, and L. Paulesu, "Role of the macrophage migration inhibitory factor (MIF) in the survival of first trimester human placenta under induced stress conditions," *Scientific Reports*, vol. 8, no. 1, 2018.

[32] H. Terheyden, B. Stadlinger, M. Sanz, A. I. Garbe, and J. Meyle, "Inflammatory reaction - communication of cells," *Clinical Oral Implants Research*, vol. 25, no. 4, pp. 399–407, 2014.

[33] L. Abusleme and N. M. Moutsopoulos, "IL-17: overview and role in oral immunity and microbiome," *Oral Diseases*, vol. 23, no. 7, pp. 854–865, 2017.

[34] V. P. B. Parachuru, D. E. Coates, T. J. Milne, A. M. Rich, and G. J. Seymour, "FoxP3$^+$ regulatory T cells, interleukin 17 and mast cells in chronic inflammatory periodontal disease," *Journal of Periodontal Research*, vol. 53, no. 4, pp. 622–635, 2018.

[35] K. Yamazaki, T. Nakajima, E. Gemmell, B. Polak, G. J. Seymour, and K. Hara, "IL-4- and IL-6-producing cells in human periodontal disease tissue," *Journal of Oral Pathology & Medicine*, vol. 23, no. 8, pp. 347–353, 1994.

[36] Y. Matsuki, T. Yamamoto, and K. Hara, "Detection of inflammatory cytokine messenger RNA (mRNA)-expressing cells in human inflamed gingiva by combined in situ hybridization and immunohistochemistry," *Immunology*, vol. 76, no. 1, pp. 42–47, 1992.

[37] N. Stein, "Position Paper:Diabetes and periodontal diseases," *Journal of Periodontology*, vol. 71, no. 4, pp. 664–678, 2000.

[38] C. F. Francisconi, A. E. Vieira, M. C. S. Azevedo et al., "RANKL triggers Treg-mediated immunoregulation in inflammatory osteolysis," *Journal of Dental Research*, vol. 97, no. 8, pp. 917–927, 2018.

[39] C. Alvarez, C. Rojas, L. Rojas, E. A. Cafferata, G. Monasterio, and R. Vernal, "Regulatory T lymphocytes in periodontitis: a translational view," *Mediators of Inflammation*, vol. 2018, Article ID 7806912, 10 pages, 2018.

[40] S. C. Liang, X. Y. Tan, D. P. Luxenberg et al., "Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides," *The Journal of Experimental Medicine*, vol. 203, no. 10, pp. 2271–2279, 2006.

[41] S. R. Lester, J. L. Bain, R. B. Johnson, and F. G. Serio, "Gingival concentrations of interleukin-23 and -17 at healthy sites and at sites of clinical attachment loss," *Journal of Periodontology*, vol. 78, no. 8, pp. 1545–1550, 2007.

[42] K. Bunte and T. Beikler, "Th17 cells and the IL-23/IL-17 axis in the pathogenesis of periodontitis and immune-mediated inflammatory diseases," *International Journal of Molecular Sciences*, vol. 20, no. 14, p. 3394, 2019.

[43] P. Miossec and J. K. Kolls, "Targeting IL-17 and T_H17 cells in chronic inflammation," *Nature Reviews Drug Discovery*, vol. 11, no. 10, pp. 763–776, 2012.

[44] M. E. L. Sommer, R. A. Dalia, A. V. B. Nogueira et al., "Immune response mediated by Th1 / IL-17 / caspase-9 promotes evolution of periodontal disease," *Archives of Oral Biology*, vol. 97, pp. 77–84, 2019.

[45] R. Jotwani and C. W. Cutler, "Multiple dendritic cell (DC) subpopulations in human gingiva and association of mature DCs with CD4+ T-cells in situ," *Journal of Dental Research*, vol. 82, no. 9, pp. 736–741, 2003.

[46] K. Hoebe, E. Janssen, and B. Beutler, "The interface between innate and adaptive immunity," *Nature Immunology*, vol. 5, no. 10, pp. 971–974, 2004.

[47] T. Kawai, T. Matsuyama, Y. Hosokawa et al., "B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease," *The American Journal of Pathology*, vol. 169, no. 3, pp. 987–998, 2006.

[48] E. Könönen, M. Gursoy, and U. K. Gursoy, "Periodontitis: a multifaceted disease of tooth-supporting tissues," *Journal of Clinical Medicine*, vol. 8, no. 8, p. 1135, 2019.

[49] A. Cappariello, A. Maurizi, V. Veeriah, and A. Teti, "_The Great Beauty_ of the osteoclast," *Archives of Biochemistry and Biophysics*, vol. 558, pp. 70–78, 2014.

[50] M. C. Walsh, N. Kim, Y. Kadono et al., "Osteoimmunology: interplay between the immune system and bone metabolism," *Annual Review of Immunology*, vol. 24, no. 1, pp. 33–63, 2006.

[51] M. A. Karsdal, K. Henriksen, M. G. Sørensen et al., "Acidification of the osteoclastic resorption compartment provides insight into the coupling of bone formation to bone resorption," *The American Journal of Pathology*, vol. 166, no. 2, pp. 467–476, 2005.

[52] W. F. Rodrigues, M. F. M. Madeira, T. A. da Silva et al., "Low dose of propranolol down-modulates bone resorption by inhibiting inflammation and osteoclast differentiation," *British Journal of Pharmacology*, vol. 165, no. 7, pp. 2140–2151, 2012.

[53] G. Luo, F. Li, X. Li, Z. G. Wang, and B. Zhang, "TNF-α and RANKL promote osteoclastogenesis by upregulating RANK

via the NF-κB pathway," *Molecular Medicine Reports*, vol. 17, no. 5, pp. 6605–6611, 2018.

[54] A. di Benedetto, I. Gigante, S. Colucci, and M. Grano, "Periodontal disease: linking the primary inflammation to bone loss," *Clinical & Developmental Immunology*, vol. 2013, article 503754, pp. 1–7, 2013.

[55] Y. T. Teng, H. Nguyen, X. Gao et al., "Functional human T-cell immunity and osteoprotegerin ligand control alveolar bone destruction in periodontal infection," *The Journal of Clinical Investigation*, vol. 106, no. 6, pp. R59–R67, 2000.

[56] M. C. Walsh and Y. Choi, "Biology of the RANKL-RANK-OPG system in immunity, bone, and beyond," *Frontiers in Immunology*, vol. 5, p. 511, 2014.

[57] Y. Esparza-Guerrero, C. A. Nava-Valdivia, A. M. Saldaña-Cruz et al., "El sistema RANK/RANKL/OPG y sus implicaciones clínicas en la osteoporosis," *El Residente*, vol. 11, no. 3, pp. 99–104, 2016.

[58] Q. Zhao, J. Shao, W. Chen, and Y. P. Li, "Osteoclast differentiation and gene regulation," *Frontiers in Bioscience*, vol. 12, pp. 2519–2529, 2007.

[59] W. Dong, J. Xiang, C. Li, Z. Cao, and Z. Huang, "Increased expression of extracellular matrix metalloproteinase inducer is associated with matrix metalloproteinase-1 and -2 in gingival tissues from patients with periodontitis," *Journal of Periodontal Research*, vol. 44, no. 1, pp. 125–132, 2009.

[60] G. S. Butler and C. M. Overall, "Matrix metalloproteinase processing of signaling molecules to regulate inflammation," *Periodontology 2000*, vol. 63, no. 1, pp. 123–148, 2013.

[61] J. Bartmann, M. Frankenberger, C. Neurohr, O. Eickelberg, E. Noessner, and W. von Wulffen, "A novel role of MMP-13 for murine DC function: its inhibition dampens T-cell activation," *International Immunology*, vol. 28, no. 10, pp. 473–487, 2016.

[62] A. L. Ejeil, S. Igondjo-Tchen, S. Ghomrasseni, B. Pellat, G. Godeau, and B. Gogly, "Expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in healthy and diseased human gingiva," *Journal of Periodontology*, vol. 74, no. 2, pp. 188–195, 2003.

[63] G. Sapna, S. Gokul, and K. Bagri-Manjrekar, "Matrix metalloproteinases and periodontal diseases," *Oral Diseases*, vol. 20, no. 6, pp. 538–550, 2014.

[64] M. Gürsoy, F. Zeidán-Chuliá, E. Könönen et al., "Pregnancy-induced gingivitis and OMICS in dentistry: in silico modeling and In Vivo prospective validation of estradiol-modulated inflammatory biomarkers," *OMICS*, vol. 18, no. 9, pp. 582–590, 2014.

[65] E. Y. Liao and X. H. Luo, "Effects of 17beta-estradiol on the expression of matrix metalloproteinase-1, -2 and tissue inhibitor of metalloproteinase-1 in human osteoblast-like cell cultures," *Endocrine*, vol. 15, no. 3, pp. 291–296, 2001.

[66] M. Nishikawa, Y. Yamaguchi, K. Yoshitake, and Y. Saeki, "Effects of TNFalpha and prostaglandin E2 on the expression of MMPs in human periodontal ligament fibroblasts," *Journal of Periodontal Research*, vol. 37, no. 3, pp. 167–176, 2002.

[67] C. Behm, M. Nemec, F. Weissinger, M. A. Rausch, O. Andrukhov, and E. Jonke, "MMPs and TIMPs expression levels in the periodontal ligament during orthodontic tooth movement: a systematic review of in vitro and in vivo studies," *International Journal of Molecular Sciences*, vol. 22, no. 13, p. 6967, 2021.

[68] C. Behm, M. Nemec, A. Blufstein et al., "Interleukin-1β induced matrix metalloproteinase expression in human periodontal ligament-derived mesenchymal stromal cells under in vitro simulated static orthodontic forces," *International Journal of Molecular Sciences*, vol. 22, no. 3, p. 1027, 2021.

[69] M. Dahan, B. Nawrocki, R. Elkaïm et al., "Expression of matrix metalloproteinases in healthy and diseased human gingiva," *Journal of Clinical Periodontology*, vol. 28, no. 2, pp. 128–136, 2001.

[70] J. Zhou, J. Zhang, and J. Chao, "Porphyromonas gingivalis promotes monocyte migration by activating MMP-9," *Journal of Periodontal Research*, vol. 47, no. 2, pp. 236–242, 2012.

[71] M. Solera, R. Jose, M. Conte et al., "Papel de las Metaloproteinasas de la Matriz en la Degradación del Tejido Pulpar: Una revisión literaria," *Revista Científica Odontológica*, vol. 1, no. 1, pp. 20–26, 2005.

[72] K. C. Nannuru, M. Futakuchi, M. L. Varney, T. M. Vincent, E. G. Marcusson, and R. K. Singh, "Matrix metalloproteinase (MMP)-13 regulates mammary tumor-induced osteolysis by activating MMP9 and transforming growth factor-beta signaling at the tumor-bone interface," *Cancer Research*, vol. 70, no. 9, pp. 3494–3504, 2010.

[73] N. Silva, L. Abusleme, D. Bravo et al., "Host response mechanisms in periodontal diseases," *Journal of Applied Oral Science*, vol. 23, no. 3, pp. 329–355, 2015.

[74] R. Hernandez-Bello, K. Nava-Castro, S. Muniz-Hernandez et al., "Beyond the reproductive effect of sex steroids: their role during immunity to helminth parasite infections," *Mini Reviews in Medicinal Chemistry*, vol. 12, no. 11, pp. 1071–1080, 2012.

[75] R. Lamason, P. Zhao, R. Rawat et al., "Sexual dimorphism in immune response genes as a function of puberty," *BMC Immunology*, vol. 7, no. 1, p. 2, 2006.

[76] F. X. LÜ, K. ABEL, Z. MA et al., "The strength of B cell immunity in female rhesus macaques is controlled by CD8+ T cells under the influence of ovarian steroid hormones," *Clinical and Experimental Immunology*, vol. 128, no. 1, pp. 10–20, 2002.

[77] Y. Fu, L. Li, X. Liu et al., "Estrogen promotes B cell activation in vitro through down-regulating CD80 molecule expression," *Gynecological Endocrinology*, vol. 27, no. 8, pp. 593–596, 2011.

[78] K. Z. Matalka, "The effect of estradiol, but not progesterone, on the production of cytokines in stimulated whole blood, is concentration-dependent," *Neuro Endocrinology Letters*, vol. 24, no. 3-4, pp. 185–191, 2003.

[79] E. Karpuzoglu-Sahin, B. D. Hissong, and S. Ansar Ahmed, "Interferon-_γ_ levels are upregulated by 17- _β_ -estradiol and diethylstilbestrol," *Journal of Reproductive Immunology*, vol. 52, no. 1-2, pp. 113–127, 2001.

[80] D. P. Robinson and S. L. Klein, "Pregnancy and pregnancy-associated hormones alter immune responses and disease pathogenesis," *Hormones and Behavior*, vol. 62, no. 3, pp. 263–271, 2012.

[81] M. C. Siracusa, M. G. Overstreet, F. Housseau, A. L. Scott, and S. L. Klein, "17beta-estradiol alters the activity of conventional and IFN-producing killer dendritic cells," *Journal of Immunology*, vol. 180, no. 3, pp. 1423–1431, 2008.

[82] T. Wang, S. D. Yang, S. Liu, H. Wang, H. Liu, and W. Y. Ding, "17β-Estradiol inhibites tumor necrosis Factor-α induced apoptosis of human nucleus pulposus cells via the PI3K/Akt

pathway," *Medical Science Monitor*, vol. 22, pp. 4312–4322, 2016.

[83] J. S. Walsh, "Normal bone physiology, remodelling and its hormonal regulation," *Surgery*, vol. 33, no. 1, pp. 1–6, 2015.

[84] S. L. Klein, "Immune cells have sex and so should journal articles," *Endocrinology*, vol. 153, no. 6, pp. 2544–2550, 2012.

[85] C. L. Butts, E. Bowers, J. C. Horn et al., "Inhibitory effects of progesterone differ in dendritic cells from female and male rodents," *Gender Medicine*, vol. 5, no. 4, pp. 434–447, 2008.

[86] L. I. McKay and J. A. Cidlowski, "Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways," *Endocrine Reviews*, vol. 20, no. 4, pp. 435–459, 1999.

[87] I. J. Tan, E. Peeva, and G. Zandman-Goddard, "Hormonal modulation of the immune system – A spotlight on the role of progestogens," *Autoimmunity Reviews*, vol. 14, no. 6, pp. 536–542, 2015.

[88] J. H. Lee, J. P. Lydon, and C. H. Kim, "Progesterone suppresses the mTOR pathway and promotes generation of induced regulatory T cells with increased stability," *European Journal of Immunology*, vol. 42, no. 10, pp. 2683–2696, 2012.

[89] S. Altuwaijri, K. H. Chuang, K. P. Lai et al., "Susceptibility to autoimmunity and B cell resistance to apoptosis in mice lacking androgen receptor in B cells," *Molecular Endocrinology*, vol. 23, no. 4, pp. 444–453, 2009.

[90] S. Aboudkhil, A. Zaîd, L. Henry, and J. P. Bureau, "Influence of age, castration, and testosterone on T cell subsets in healthy and leukemia grafted mice," *Biology of the Cell*, vol. 95, no. 1, pp. 9–16, 2003.

[91] J. J. Lai, K. P. Lai, K. H. Chuang et al., "Monocyte/macrophage androgen receptor suppresses cutaneous wound healing in mice by enhancing local TNF-alpha expression," *The Journal of Clinical Investigation*, vol. 119, no. 12, pp. 3739–3751, 2009.

[92] M. K. Angele, M. W. Knöferl, A. Ayala, K. I. Bland, and I. H. Chaudry, "Testosterone and estrogen differently effect Th1 and Th2 cytokine release following trauma-haemorrhage," *Cytokine*, vol. 16, no. 1, pp. 22–30, 2001.

[93] E. Posma, H. Moes, M. J. Heineman, and M. M. Faas, "The effect of testosterone on cytokine production in the specific and non-specific immune response," *American Journal of Reproductive Immunology*, vol. 52, no. 4, pp. 237–243, 2004.

[94] S. A. Ahmed, E. Karpuzoglu, and D. Khan, *Effects of sex steroids on innate and adaptive immunity, in Sex hormones and immunity to infection*, Springer, 2010.

[95] J. J. Corrales, M. Almeida, R. Burgo, M. T. Mories, J. M. Miralles, and A. Orfao, "Androgen-replacement therapy depresses the ex vivo production of inflammatory cytokines by circulating antigen-presenting cells in aging type-2 diabetic men with partial androgen deficiency," *The Journal of Endocrinology*, vol. 189, no. 3, pp. 595–604, 2006.

[96] N. Kanda, T. Tsuchida, and K. Tamaki, "Testosterone inhibits immunoglobulin production by human peripheral blood mononuclear cells," *Clinical and Experimental Immunology*, vol. 106, no. 2, pp. 410–415, 1996.

[97] B. L. Clarke and S. Khosla, "Androgens and bone," *Steroids*, vol. 74, no. 3, pp. 296–305, 2009.

[98] K. M. Wiren, "Androgens and bone growth: it's location, location, location," *Current Opinion in Pharmacology*, vol. 5, no. 6, pp. 626–632, 2005.

[99] S. Kovats, "Estrogen receptors regulate innate immune cells and signaling pathways," *Cellular Immunology*, vol. 294, no. 2, pp. 63–69, 2015.

[100] S. Kovats, E. Carreras, and H. Agrawal, *Sex steroid receptors in immune cells, in Sex hormones and immunity to infection*, Springer, 2010.

[101] L. A. Cervantes-Candelas, J. Aguilar-Castro, F. O. Buendía-González et al., "17β-Estradiol is involved in the sexual dimorphism of the immune response to malaria," *Frontiers in Endocrinology*, vol. 12, article 643851, 2021.

[102] X. Duan, A. Hays, W. Zhou et al., "_Porphyromonas gingivalis_ induces exacerbated periodontal disease during pregnancy," *Microbial Pathogenesis*, vol. 124, pp. 145–151, 2018.

[103] C. Ye, Z. Xia, J. Tang et al., "Unculturable and culturable periodontal-related bacteria are associated with periodontal inflammation during pregnancy and with preterm low birth weight delivery," *Scientific Reports*, vol. 10, no. 1, article 15807, 2020.

[104] D. Tulchinsky, C. J. Hobel, E. Yeager, and J. R. Marshall, "Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy: I. Normal pregnancy," *American Journal of Obstetrics and Gynecology*, vol. 112, no. 8, pp. 1095–1100, 1972.

[105] S. S. Soldan, A. I. A. Retuerto, N. L. Sicotte, and R. R. Voskuhl, "Immune modulation in multiple sclerosis patients treated with the pregnancy hormone estriol," *Journal of Immunology*, vol. 171, no. 11, pp. 6267–6274, 2003.

[106] G. M. Knight and A. Bryan Wade, "The effects of hormonal contraceptives on the human periodontium," *Journal of Periodontal Research*, vol. 9, no. 1, pp. 18–22, 1974.

[107] K. S. Kornman and W. J. Loesche, "The subgingival microbial flora during pregnancy," *Journal of Periodontal Research*, vol. 15, no. 2, pp. 111–122, 1980.

[108] R. S. S. Massoni, A. M. F. Aranha, F. Z. Matos et al., "Correlation of periodontal and microbiological evaluations, with serum levels of estradiol and progesterone, during different trimesters of gestation," *Scientific Reports*, vol. 9, no. 1, article 11762, 2019.

[109] P. S. Kumar, "Sex and the subgingival microbiome: do female sex steroids affect periodontal bacteria?," *Periodontology 2000*, vol. 61, no. 1, pp. 103–124, 2013.

[110] L. Liang, J. F. Yu, Y. Wang, and Y. Ding, "Estrogen regulates expression of osteoprotegerin and RANKL in human periodontal ligament cells through estrogen receptor beta," *Journal of Periodontology*, vol. 79, no. 9, pp. 1745–1751, 2008.

[111] M. G. Rico-Rosillo and G. B. Vega-Robledo, "Immunological mechanisms involved in pregnancy," *Ginecología y Obstetricia de México*, vol. 80, no. 5, pp. 332–340, 2012.

[112] G. Mor, P. Aldo, and A. B. Alvero, "The unique immunological and microbial aspects of pregnancy," *Nature Reviews Immunology*, vol. 17, no. 8, pp. 469–482, 2017.

[113] R. Raghupathy, "Pregnancy: success and failure within the Th1/Th2/Th3 paradigm," *Seminars in Immunology*, vol. 13, no. 4, pp. 219–227, 2001.

[114] P. Le Bouteiller and A. Bensussan, "Up-and-down immunity of pregnancy in humans," *F1000Research*, vol. 6, p. 1216, 2017.

[115] J. Qian, N. Zhang, J. Lin et al., "Distinct pattern of Th17/Treg cells in pregnant women with a history of unexplained recurrent spontaneous abortion," *Bioscience Trends*, vol. 12, no. 2, pp. 157–167, 2018.

[116] W. Wang, N. Sung, A. Gilman-Sachs, and J. Kwak-Kim, "T helper (Th) cell profiles in pregnancy and recurrent pregnancy losses: Th1/Th2/Th9/Th17/Th22/Tfh cells," *Frontiers in Immunology*, vol. 11, p. 2025, 2020.

[117] M. Morishita, M. Miyagi, and Y. Iwamoto, "Effects of sex hormones on production of interleukin-1 by human peripheral monocytes," *Journal of Periodontology*, vol. 70, no. 7, pp. 757–760, 1999.

[118] V. Brazão, C. C. Kuehn, C. D. . Santos, C. M. B. . Costa, J. C. . P. Júnior, and A. A. Carraro-Abrahão, "Endocrine and immune system interactions during pregnancy," *Immunobiology*, vol. 220, no. 1, pp. 42–47, 2015.

[119] S. M. Blois, R. Joachim, J. Kandil et al., "Depletion of CD8+ cells abolishes the pregnancy protective effect of progesterone substitution with dydrogesterone in mice by altering the Th1/Th2 cytokine profile," *Journal of Immunology*, vol. 172, no. 10, pp. 5893–5899, 2004.

[120] A. Mariotti and M. Mawhinney, "Endocrinology of sex steroid hormones and cell dynamics in the periodontium," *Periodontology 2000*, vol. 61, no. 1, pp. 69–88, 2013.

[121] N. Urteaga Mamani, W. Challapa Licidio, and J. Cortez, "Inmunología de la Implantación," *Revista SCientifica*, vol. 4, p. 48, 2006.

[122] F. Q. Bui, C. L. C. Almeida-da-Silva, B. Huynh et al., "Association between periodontal pathogens and systemic disease," *Biomedical Journal*, vol. 42, no. 1, pp. 27–35, 2019.

[123] M. Wu, S. W. Chen, W. L. Su et al., "Sex Hormones Enhance Gingival Inflammation without Affecting IL-1β and TNF-α in Periodontally Healthy Women during Pregnancy," *Mediators of Inflammation*, vol. 2016, Article ID 4897890, 6 pages, 2016.

[124] H. Ren and M. Du, "Role of maternal periodontitis in preterm birth," *Frontiers in Immunology*, vol. 8, p. 139, 2017.

[125] S. Komine-Aizawa, S. Aizawa, and S. Hayakawa, "Periodontal diseases and adverse pregnancy outcomes," *The Journal of Obstetrics and Gynaecology Research*, vol. 45, no. 1, pp. 5–12, 2019.

[126] C. Silva de Araujo Figueiredo, C. Gonçalves Carvalho Rosalem, A. L. Costa Cantanhede, É. B. Abreu Fonseca Thomaz, and M. C. Fontoura Nogueira da Cruz, "Systemic alterations and their oral manifestations in pregnant women," *Journal of Obstetrics and Gynaecology Research*, vol. 43, no. 1, pp. 16–22, 2017.

[127] A. Hays, X. Duan, J. Zhu et al., "Down-regulated Treg cells in exacerbated periodontal disease during pregnancy," *International Immunopharmacology*, vol. 69, pp. 299–306, 2019.

[128] I. Cetin, P. Pileri, A. Villa, S. Calabrese, L. Ottolenghi, and S. Abati, "Pathogenic mechanisms linking periodontal diseases with adverse pregnancy outcomes," *Reproductive Sciences*, vol. 19, no. 6, pp. 633–641, 2012.

[129] J. Y. Kwak-Kim, H. S. Chung-Bang, S. C. Ng et al., "Increased T helper 1 cytokine responses by circulating T cells are present in women with recurrent pregnancy losses and in infertile women with multiple implantation failures after IVF," *Human Reproduction*, vol. 18, no. 4, pp. 767–773, 2003.

[130] S. Offenbacher, S. Lieff, K. A. Boggess et al., "Maternal periodontitis and prematurity. Part I: obstetric outcome of prematurity and growth restriction," *Annals of Periodontology*, vol. 6, no. 1, pp. 164–174, 2001.

[131] C. M. Cobb, P. Kelly, K. Williams, S. Babbar, M. Angolkar, and R. Derman, "The oral microbiome and adverse pregnancy outcomes," *International Journal of Women's Health*, vol. Volume 9, pp. 551–559, 2017.

[132] M. González-Jaranay, L. Téllez, A. Roa-López, G. Gómez-Moreno, and G. Moreu, "Periodontal status during pregnancy and postpartum," *PLoS One*, vol. 12, no. 5, article e0178234, 2017.

[133] M. F. Fogacci, E. . O. C. Cardoso, D. . S. Barbirato, D. P. de Carvalho, and C. Sansone, "No association between periodontitis and preterm low birth weight: a case-control study," *Archives of Gynecology and Obstetrics*, vol. 297, no. 1, pp. 71–76, 2018.

[134] A. P. C. G. Seraphim, F. Y. Chiba, R. F. Pereira, M. S. . L. C. Mattera, S. A. S. Moimaz, and D. H. Sumida, "Relationship among periodontal disease, insulin resistance, salivary cortisol, and stress levels during pregnancy," *Brazilian Dental Journal*, vol. 27, no. 2, pp. 123–127, 2016.

[135] L. A. Daalderop, B. V. Wieland, K. Tomsin et al., "Periodontal disease and pregnancy outcomes: overview of systematic reviews," *JDR Clinical & Translational Research*, vol. 3, no. 1, pp. 10–27, 2018.

[136] G. A. Foratori-Junior, B. M. da Silva, A. C. da Silva Pinto, H. M. Honório, F. C. Groppo, and S. H. de Carvalho Sales-Peres, "Systemic and periodontal conditions of overweight/-obese patients during pregnancy and after delivery: a prospective cohort," *Clinical Oral Investigations*, vol. 24, no. 1, pp. 157–165, 2020.

14 Anexo III Técnicas

14.1 Inmunofluorescencia en secciones embebidas en parafina tejidos óseos y dentales

- Fijación de tejido.

1. Tejido diseccionado recientemente mayor a 3 mm, fijar con paraformaldehído por 30 minutos, lavar durante 30 minutos con agua corriente en agitación, con cambio de agua cada 5 minutos.

- Decalcificación

2. Colocar el tejido en solución fresca de EDTA 7% (20 volúmenes), realizando cambios cada 48 hrs, hasta que el tejido tenga una consistencia blanda (durante 18 días), después lavar en agua corriente 1 hr, en agitación, con cambio de agua cada 5 minutos.

H20 destilada	80 mL
EDTA	7 g

Agitar y adicionar NAOH1N hasta que se disuelva el EDTA, (calentar ligeramente)

Ajustar pH con HCL IN a 7.4 y aforar a 100 mL

- Deshidratación

3. Pasar las muestras por el tren de deshidratación

Alcohol 60%	40 min
Alcohol 70%	40 min
Alcohol 80%	40 min
Alcohol 90%	40 min
Alcohol 96%	40 min
Alcohol Amílico	20 min
Aceite de cedro	48 hrs

- Inclusión

4. Colocar las muestras en canastillas de inclusión, y pasarlas en parafinas, temperatura máxima 58°C

Parafina – Aceite	12 hrs
Parafina I	12 hrs
Parafina II	12 hrs

Inclusión en Paraplast (Verificar la posición del tejido para que los cortes sean coronales)

- Preparación de portaobjetos

5. Colocar los portaobjetos nuevos en solución de Poly L-lisina, en refrigeración 4°C durante 24 hrs, secar tapados durante 24 hrs, guardar.

Poly L-lisina	10 ml
Agua Mq	90 ml

- Preparación de Ruyter

6. Se utiliza para ayudar a estirar las muestras en cama de estiramiento

Adicionar albumina al agua destilada, en agitación

Disolver el benzoato (gota a gota lentamente) en la acetona (en agitación leve)

Mezclar ambas soluciones gota a gota muy lentamente y después filtrar

Acetona	20 ml
Benzoato de metilo	20 gotas
Albumina glicerinada	20 gotas
Agua destilada	80 ml

14.2 Tinción H&E

Xilol I	5 min
Xilol II	5 min
Alcohol etílico absoluto	5 min
Alcohol etílico 96%	5 min
Alcohol etílico 80%	5 min
Alcohol etílico 70%	5 min
Agua destilada	5 min
Hematoxilina de Harris	5 min
Agua corriente	10 seg
Alcohol ácido: Alcohol al 70% + 1% de HCl	10 seg (chorreo con pipeta Pasteur)
Agua destilada	Enjuagar
Agua amoniacal: $H2O$ destilada + 1% hidróxido de amonio	30 seg
Agua destilada	Enjuagar
Eosina	2 min
Agua destilada	Enjuagar
Alcohol etílico 96%	Lavar por chorreo con pipeta Pasteur
Alcohol etílico absoluto	30 seg
Xilol	5 min

Montar con resina entellán (Merck)

14.3 Tinción tricrómica de Masson (HT15-KT Sigma-Aldich)

Xilol I	5 min
Xilol II	5 min

Alcohol etílico absoluto	5 min
Alcohol etílico 96%	5 min
Alcohol etílico 80%	5 min
Alcohol etílico 70%	5 min
Agua destilada	5 min
Bowin	48hrs temperatura ambiente
Agua corriente (x2)	2.5 min (enjuagar en agitación)
Hematoxilina de Weigert	8 min
Agua destilada	5 min
Rojo escarlata (Sigma-Adlrich)	8 min
Agua destilada	5 min
Ácido fosfomolíbdico/fosfotúngstico (Sigma-Aldrich)	8 min
Azul anilina (Sigma-Aldrich)	8 min
Ácido acético	3 min (cambiar cada uso)
Alcohol etílico 70%	30 seg
Alcohol etílico 80%	30 seg
Alcohol etílico 96%	30 seg
Alcohol etílico absolute	15 seg
Xilol	5 min

Montar con resina entellán (Merck)

14.4 Inmunofluorescencia

7. Técnica para laminillas montadas en parafina, técnica indirecta

Porta objetos poly-L-lisinizados o gelatinizados

Los cortes de tejidos deben ser entre 3 y 5 micras (Parafina)

Desparafinar	Toda la noche
Xilol I	5 minutos
Xilol II	5 minutos
Alcohol 100%	1 minuto
Alcohol 90%	1 minuto
Alcohol 80%	1 minuto
Alcohol 70%	1 minuto
Agua Destilada	3 minutos

<u>*Cámara Húmeda</u>

PBS 1X (3 Veces)	1 minuto
PBS TRITON X-100 (0.5%)	5 minutos (500µl Tritón + 99.5ml PBS)
PBS 1X (2 Veces)	1 minuto
Albúmina sérica bovina 1X en PBS	120 MINUTOS (2 hrs)
10 mg albumina + 90 ml PBS	
PBS TRITON X-100 (0.5%)	5 minutos (3 veces)
PBS 1X (2 Veces)	1 minuto

Para dos anticuerpos primarios:

Incubar con 1er anticuerpo 1:500	2 hrs a 36°C
50µl/muestra coverplate	30 µl/ muestra parafilm
PBS 1X (8 Veces)	1 minuto
Incubar con 2do 1er anticuerpo 1:500	2 hrs a 36°C
50µl/muestra coverplate	30 µl/ muestra parafilm

***Obscuridad**

Incubar con 2dos anticuerpo Fluoresceínado 1:1500 + HOTCH 1:200 2 hrs a 36°C (Toda la noche a 4°C)

PBS 1X (2 Veces)	1 minuto
Alcohol 70%	1 minuto
Alcohol 80%	1 minuto
Alcohol 90% (2 veces)	1 minuto
Alcohol 100%	5 minutos
Alcohol-Xilol	5 minutos
Xilol I	5 minutos
Xilol II	1 minuto

Montar con Entellan y guardar en la obscuridad, obtener imágenes con microscopio Confocal, analizar en Leica Application Suite X

14.5 Extracción de RNA muestras de maxilar

1. Los tejidos se lavan en PBS y se trituran en nitrógeno líquido, se colocan en un tubo Eppendorf con 200 µL de Trizol (Ambion Ref. 15596018). Mantener en frio y dejar a -70° mínimo 48 hrs hasta 1 mes.
2. En esterilidad, agregar 40 µL de cloroformo grado molecular por cada 200 µL de trizol y mezclar en el vórtex.
3. Incubar a temperatura ambiente 10 minutos. (5 minutos)
4. Centrifugar a 14,000 rpm por 15 minutos a 4°C.
5. Después de centrifugar quedan tres fases. Tomar solamente la capa superior (transparente) el sobrenadante, colocar en tubos Eppendorf nuevos, agregar 50 µL de isopropanol frío y mezclar en el vórtex de forma ligera. Invertir y revertir 5 segundos. (tirar los residuos en contenedor de trizol)
6. Incubar a temperatura ambiente por 10 minutos.

7. Centrifugar a 14,000 rpm durante 15 minutos a 4°C
8. Tirar el sobrenadante y resuspender con 500 µL de etanol al 75%, mezclar en el vórtex de forma ligera (verificar que se despegue la pastilla).
9. Centrifugar a 10,000 rpm por 5 minutos a 4°C. Retirar etanol con pipeta.
10. Repetir pasos 8 y 9
11. Secar el botón a temperatura ambiente 15 – 20 minutos (máximo 30). Seco resuspender con 20 µL de H_2O DEPC y agregar inhibidor 1µL por tubo (botón).
12. Colocar en el termoblock ya resuspendido a 37°C.
13. Cuantificar en espectrofotómetro a 260 nm. (EPOCH o Nanodrop)

14.6 **Obtención de cDNA a partir de RNA (SuperScript First -Strand Invotrogen 11904-018)**

1. Tomar los µL resultantes de las muestras en los tubos para PCR y ajustar a 2000 ng/mL de RNA en 8 µL.

Agregar:

1) 1 µL de dNTP's
2) 1 µL de OligoDT

2. Aforar a 10 µL con H_2O.
3. Colocar en el termociclador con el programa de cDNA
4. 65°C por 5 minutos.
5. 4° C por 1 minuto
6. Preparar (RT-PCR Invitrogen) la siguiente reacción por cada muestra:
    3) 2 µL de Buffer RT
    4) 4 µL de MgCl
    5) 2 µL de DTT
    6) 1 µL de RNA OUT

    Poner 9 µL a cada muestra
7. 42°C por 2 minutos.
8. 4°C por 1 minuto. Durante este tiempo agregar

9. 0.5 µL de SSIIRT.
10. 42°C por 50 minutos.
11. 70°C por 15 minutos.
12. 4°C durante 1 minuto. Durante este tiempo agregar
13. 0.5 µL de RNAsa H.
14. 36°C por 15 minutos.
15. Agregar 1 µl de Inhibidor de RNAsas (Invitrogen)
16. Cuantificar el cDNA obtenido, y preparar alícuotas de trabajo de 100µg/µl
17. Almacenar a -70°C.

14.7 RT-PCR punto final (ADN polimerasa Taq, Thermo Scientific EP0402)

Preparar la siguiente reacción por cada muestra: *Los primers a utilizar corresponden a la secuencia del gen que se quiere cuantificar en cada reacción.

- Buffer 2.5 µL
- dNTP's 0.6 µL
- MgCl 1.3 µL
- Primer Forward* 1 µL
- Primer Reverse* 1 µL
- H_2O 16.1 µL
- Taq 0.5 µL
- cDNA 2 µL
- Volumen total 20 µl

1. Colocar en el termociclador con el programa de PCR que corresponde a los gradientes de temperatura de amplificación de los genes (Tabla 1) Paso 3.

   Programa Termociclador:

   Lid: 105 °C

   Volumen: 20 µl

   1) 95 °C, 5:00
   2) 95 °C, 0:35
   3) 57 °C, 0:35 Esta temperatura se ajusta según el primer del gen
   4) 72 °C, 0:35

5) GOTO step 2, 35X
6) 72 °C, 5:00
7) 4 °C, 10:00

2. Preparar gel de agarosa a 1.5%. (Gel grande: 40mL TBE, 0.60g agarosa, Bromuro de etidio 2µl/15mL TBE)
3. Cuando el termociclador ha terminado, colocar Blue Juke (6 µL) a todas las muestras.
4. Cargar las muestras y el marcador de peso molecular en el gel de bromuro de etidio.
5. Realizar electroforesis durante 45 minutos.
6. Leer en el fotodocumentador y realizar la cuantificación con el programa Biorad Image Lab.

14.8 Thermo Scientific ReveseAid First Strand cDNA Synthesis Kit K1622

Después de extraer y cuantificar el RNA hacer alícuotas de 20µl con 100 ng/µl

1. Colocar 11µl de RNA a 100ng/µl
   1) Primer 1 µl
   2) Incubar a 65°C por 5 minutos
   3) 5X Reaction buffer 4 µl
   4) RiboLock RNase inhibitor 1 µl
   5) 10nM dNTP's mix 2 µl
   6) RevertAid 1 µl
2. Incubar a 42 °C por 60 minutos
   70 °C por 5 minutos
   4 °C por 5 minutos
3. Agregar 10 µl de H2O DEPC
4. Cuantificar y hacer alícuotas de 100ng/µl

14.9 qPCR (PowerSYBR Green PCR Master Mix, Applied biosystems 4367659)

Las muestras se corren por duplicado + 1 neg (H2O), 1 extra en placas para qPCR (96 Fast PCR-Platte Vollrand Sarsted 72.1980.202) sellado con Sealing Tape, optically clear, Sarsted

95.1994) o tubos (TempAssure 0.1 mL PCR 8-tube Strips, Att. Optical Caps USA Scientific 1402-2300)

*Los primers a utilizar corresponden a la secuencia del gen que se quiere cuantificar en cada reacción. Mantener en obscuridad o protegido de luz directa.

β-actina 200 mMol

1) Mix Syber green 5 µl
2) Primer Forw/Rev* 1 µl
3) H_2O 1 µl
4) Muestra 3 µl (300ng)

Volumen total 10 µl

Primers a 100 mMol

1) Mix Syber green 5 µl
2) Primer Forward* 1 µl
3) Primer Reverse* 1 µl
4) Muestra 3 µl (300ng)

Volumen total 10 µl

Las placas o tiras de tubos se centrifugan para eliminar burbujas, todo se debe mantener en frio (4°C) en placa refrigerada o hielo y en obscuridad, NO tocar la tapa de los tubos o la tira de sellado, llevar cubierto de la luz al termociclador para tiempo real. Ajustar la temperatura según el primer

Colocar en el termociclador con el programa de qPCR que corresponde a los gradientes de temperatura de amplificación de los genes (Tabla 1) Paso 3.

Programa Termociclador tiempo real:

Method: Calc
Lid: 105 °C
Volumen: 10 µl

1) 50°C, 2:00
2) 95°C, 10:00
3) 95°C, 0:15
4) 60°C, 1:00 Esta temperatura se ajusta según el primer del gen

5) Plateread
6) Goto 3, 35X
7) 65°C, 0:31
8) 65°C, 0:05
9) +05°C/Cycle
10) Ramp 0.5°C/s
11) Plateread
12) Goto 8, 60X
13) 4°C, 0.30

Leer el archivo y analizar en BioRadCFXManager

14.10 Técnica Obtención de Proteínas para Zimograma en colágena y gelatina para maxilar

Preparación de tejidos.

1. Los animales se anestesian en cámara de cloroformo o pentobarbital sódico 50 mg /kg.
2. Perfundir a través del ventrículo izquierdo con PBS o solución fisiológica (150 ml) frío, con punzocat 0.72x20mm y equipo de volúmenes medidos de 100ml.
3. Obtener el maxilar, lavar en PBS frio, retirar los excedentes de tejido, y macerar por criofractura en mortero con nitrógeno líquido, agregar 30µl de solución RIPA con inhibidor de proteasas sin EDTA (1µL /100µl) a 4°C

RIPA

Agua Bidestilada	10 ml	50 ml	100ml
Tris HCL	5 mg (0.005g)	25 mg (0.25g)	50 mg (0.05g)
NaCl	15 mg (0.015g)	75 mg (0.075g)	150mg (0.15g)
SDS	0.1 g	0.5 g	1 g
Deoxicolato de Na	0.05 g	0.25 g	0.5 g
Triton X100	0.1 ml	0.5 ml	1 ml

Inhibidor de proteasas 1X: 1µl/99µl (1:100) [1ml 990µl RIPA + 10µl inhibidor] (Protease Inhibitor cocktail Animal component Free Sigma-Aldrich Cat. 13786)

4. Colocar en tubos Eppendorf con 54 µL de RIPA + inhibidor mantener agitación moderada durante la incubación, evitar contaminación manteniendo condiciones estériles de soluciones y manipulando en condiciones asépticas.
5. Realizar la sonicación de la muestra durante 1 minuto con tres pulsos de 15 segundos a una amplitud de 35% y reposo de 15 segundos entre pulsos, manteniendo las muestras en hielo y evitando se forme espuma, después vortexear.
6. Dejar en agitación leve en frio durante 1 hora.
7. Centrifugar a 13 500 rpm durante 15 minutos, en frio 4°C, el sobrenadante constituye la fracción soluble en Triton X100 y el sedimento la fracción insoluble, se recupera el sobrenadante (54 – 60 µl) en tubos Eppendorf nuevos, separar 10 µl para hacer la cuantificación, mantener a -20°C, el resto se mantiene a -70°C hasta su utilización.

Bradford

1. Stock de Albumina 1:10 (0.1g/ml).
2. Diluir una vez más el Stock de Albumina 1:10 (10mg/ml).
3. Curva: En una placa para Elisa de 96 pozos, realizar en los primeros 2 carriles la curva por duplicado
4. Del Stock de albumina 10mg/ml, colocar 200 µl en el primer pozo, colocar 100 µl de agua destilada o PBS en los pozos 2 al 12 (blanco), pasar de manera seriada 100 µl del pozo 1 al 2, mezclar, tomar 100 µl y pasarlos al 3, y así sucesivamente hasta el pozo 11, tirar los últimos 100 µl.
5. Asignar los pozos que van a se utilizados para las muestras: en el primer carril colocar 95 µl de agua destilada o PBS con una pipeta multicanal, para después completar 100 µl con 5 µl de muestra. (1:20)
6. Colocar 50 µl en cada una de las filas para las diluciones seriadas 1:2.

7. Agregar los 5 µl de muestra en el primer carril y trasferir 50 µl a la fila siguiente, y así sucesivamente 3 veces, para que queden 4 filas: 1:20, 1:40, 1:80, 1:160, en la última fila el volumen final será de 100 µl.
8. Colocar el reactivo de Bradford en una canaleta y agregar 100 µl a cada pozo con una pipeta multicanal.
9. Eliminar las burbujas.
10. Cubrir de la luz y leer en el Epoch a 595nm.
11. Recuperar el archivo de D.O y analizar.

Geles de poliacrilamida

1. Preparar reactivos

- **Solución de acrilamida-bis-acrilamida (30:0.8)**

Disolver 30 g de acrilamida y 0.8 g de bis-acrilamida en 50 ml de H_2O destilada y aforar a 100 ml. Filtrar y guardar a 4°C en frasco ámbar hasta 1 mes

- **Persulfato de amonio al 1.5%**

Disolver 0.15 g de persulfato de amonio en 10 ml de H_2O destilada, filtrar y separar en alícuotas de 1.5 ml cada una Preparar antes de usar, proteger de la luz, guardar a -20°C.

- **SDS al 10% p/v**

Disolver 10 g de SDS en 70 ml de H_2O destilada, mezclar con agitador magnético hasta que se haga clara, filtrar y aforar a 100 ml. Mantener a temperatura ambiente.

- **Buffer del gel concentrador (Tris-HCl 0.5M pH 6.8).**

Disolver 6 g de Tris base en 40 ml de H_2O destilada, titular a pH de 6.8 con HCl 1 M y aforar a 100 ml, filtrar y almacenar a 4°C.

- **Buffer del gel separador (Tris-HCl 1.5 M pH 8.8).**

Disolver 18.17 g de Tris base en 48 ml de agua destilada, ajustar el pH a 8.8 con HCl 1 M y aforar a 100 ml con agua destilada, filtrar y almacenar a 4°C.

- **Buffer del reservorio (De corrida).**

    a. Solución Madre: (Tris 0.25 M, glicina 1.92 M, SDS 1% pH 8.3)

Disolver 30.3 g de Tris base, 144 g de glicina y 10 g de SDS en 800 ml de H_2O destilada, ajustar pH a 8.3 con HCl 1 M y aforar a 1000 ml con agua destilada, almacenar a 4°C.

    b. Solución de trabajo: Para su uso, diluir la solución madre 1:10 (se puede reutilizar hasta 5 veces)

- **Buffer de muestra 4X no reductor (Buffer de Carga).**

a. Solución Base

Disolver 3.03 g de Tris-HCl, en 50 ml H_2O destilada, después calentar ligeramente y separar en 2 volúmenes: 30 ml para disolver 8 g de SDS (mantener ligeramente caliente) y 20 ml para disolver 40 ml de glicerol, una vez disueltos mezclar ambas soluciones, ajustar el pH a 6.8 con HCl y aforar a 100 ml con H_2O destilada. Esta será la solución base.

b. Buffer 4X no reductor

Se emplea para las muestras de Zimograma, tres partes de amortiguador y una parte de la muestra.

Mezclar 8 ml de la solución base, con 1.6 ml de H_2O destilada y 0.4 ml de azul de bromofenol al 0.5% (50 mg (0.05g) en 10 ml de H_2O destilada.

- **Gelatina (50 mg/10 ml) (0.05 g/10 ml)** (Sigma-Aldrich Cat. G6650)

Disolver 50 mg de gelatina en 10 ml de H_2O destilada, calentar ligeramente alrededor de 40 °C en un baño de agua caliente.

- **Colágena (2 mg/2 ml) (0.002 g/ 2 ml)** (Sigma-Aldrich Cat. C9791)

Disolver 2 mg de colágena en 2 ml de Ácido Acético 0.1M, agitar vigorosamente a temperatura ambiente durante 1 – 3 hrs.

Ac acético 0.1M (5.9ml en .994ml)

2. Preparar las placas de vidrio de 1.5 mm de grosor, montar verificando que los vidrios estén paralelos en la base, con los vidrios grandes al fondo y el delgado en el frente,

dejando la pestaña con el nombre hacia arriba, ajustar y acomodar las almohadillas en la base y fijar con cuidado. Verificar que no se escurra con etanol, decantar y dejar secar.

3. Preparar el gel separador al 10% y vaciarlo en las placas, hasta la zona verde de la base aproximadamente 1 cm del borde del vidrio delgado, esperar a que polimerice, para gelatina, calentar ligeramente a 35°C acrilamida y Tris pH8.8.

Concentración 10%	1 gel gelatina	2 geles gelatina	1 gel colágena	2 geles colágena
Acrilamida 30:08	3.3 ml	6.6 ml	3.3 ml	6.6 ml
Tris pH 8.8	2.5 ml	5 ml	2.5 ml	5 ml
SDS 10%	100 µL	200 µL	100 µL	200 µL
H_2O destilada	1.42 mL	2.84 mL	2.42 mL	4.84 mL
Persulfato de amonio al 1.5%	0.66 mL	1.32 mL	0.66 mL	1.32 mL
TEMED	20 µL	40 µL	20 µL	40 µL
Gelatina /colágena (se prepara un gel de cada una)	2 ml	4 ml	1 ml	2 ml
Volumen total	10 mL	20mL	10 mL	20mL

4. Colocar isopropanol sobre el gel para eliminar las burbujas, dejar que se polimerice, decantar, lavar con agua destilada 3 veces y secar con papel filtro para quitar el excedente.

5. Preparar el gel concentrador al 3.7% y vaciarlo en las placas:

	1 gel	2 geles	3 geles
Acrilamida 30:08	0.67 ml	1.26 ml	1.89ml
Tris pH 6.8	1.25 ml	2.5ml	3.75 ml
SDS 10%	50 µl.	100 µl.	150 µl.
H_2O destilada	2.59 ml	5.18 ml	7.77 ml
Persulfato de amonio al 1.5%	333 µL	500 µL	700 µL
TEMED	10 µL	10 µL	15 µL
Volumen total	5 mL	10 L	15 mL

6. Se termina de llenar el espacio entre las placas de vidrio con el gel concentrador y se inserta la peineta, evitando dejar burbujas de aire, verificar que sea del tamaño del vidrio, así como el número y tamaño de los pozos. Se deja polimerizar 30 min.

7. Marcar con plumón indeleble la forma de los pozos por la parte externa (vidrio grueso), usar la guía de carga, apoyarla sobre el centro de los vidrios.

8. Se monta la cámara de electroforesis, se desmontan los vidrios, y se colocan en los vidrios en las monturas de los electrodos, acomodándolos con las bases hacia la parte inferior y los vidrios gruesos hacia la parte externa, se fijan y se introducen verificando la polaridad y ajustando la base, se llena la cámara interna con buffer de corrida nuevo y frio (4°C).
9. La cámara externa puede ser buffer usado (máximo 3-4 veces) se retira la peineta, se lavan los pozos con el amortiguador de reservorio (Buffer de corrida) nuevo y se llenan, se remueven las burbujas del gel lavando con buffer nuevo cada pozo con una pipeta de 1 mL.
10. Las muestras cuantificadas (Bradford) se mezclan con Buffer 4X no reductor 3 partes + 1 partes de muestra, cada pozo puede contener hasta 33 µL.
11. Simultáneamente se corren estándares de peso molecular de referencia PageRuler Prestained Protein Ladder (Thermo Cat 26616) (3µL).
12. Se llena el recipiente superior con el mismo amortiguador, se cargan las muestras en los pozos utilizando una microjeringa Hamilton o una micropipeta y puntas para geles. Cargar en orden derecha-izquierda gel exterior, izquierda-derecha gel interior.
13. Se conectan los electrodos a la fuente de poder, se corre la electroforesis a corriente constante, aplicando 80 Volts durante 30 minutos y a 90 volts durante 2 horas más, hasta que el colorante (azul de bromofenol) alcance y salga del borde inferior del gel, verificar también que el marcador de peso molecular, banda roja (70 kDa) y azul inferior (50 kDa), se encuentren a la mitad del gel separador, verificar que el tanque interno se mantenga lleno, si no rellenar con buffer nuevo.

Revelado de zimogramas.

1. Los geles se lavan dos veces en Triton X-100 al 2.5% por 30 minutos a 37°C, para remover el SDS.
2. La actividad enzimática se revela incubando durante 48 horas a 37°C en amortiguador revelador.

a. Amortiguador Revelador para 100mL

- 50nM Tris HCL 7.5 pH 0.788g / 100mL
- 10 nM $CaCl_2$ 0.11098g/ 100mL
- 150 mM NaCl 0.8766g/ 100mL
- 0.5% Triton X100 0.5mL

b. Amortiguador Inhibidor para 100mL

- 50nM Tris HCL 7.5 pH 0.788g / 100mL
- 10 nM $CaCl_2$ .11098g/ 100mL
- 150 mM NaCl 0.8766g/ 100mL
- 0.5% Triton X100 0.5mL
- 10 mM EDTA .292 g/ 100mL

Tinción Zimograma

Se tiñen con azul de comassie R-250 al 0.5% en solución desteñidora, para el gel de gelatina, dejar 5 – 10 minutos

Se tiñen con rojo sirio al 0.5 % en ácido acético, para el gel de colágena dejar 5 – 10 minutos

Las enzimas que degradan el sustrato se identifican como zonas blancas de lisis en el fondo azul o rojo.

Solución Desteñidora

	1 L	500 ml
Metanol	500mL	250
Ac. Acético Glacial	100mL	50
H2O Destilada	400mL	200

Análisis densitométrico:

Los zimogramas se capturan en un analizador de geles, y la zona de actividad enzimática es valorada por densitometría, usando el software del aparato. La cantidad de enzima se expresa en unidades arbitrarias (AU) proporcionadas por el aparato.