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TESIS DE DOCTORADO

ACCURACY OF MOLECULAR BIOMARKERS IN ORAL FLUIDS FOR DIAGNOSIS OF PERIODONTITIS

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Accuracy of Molecular Biomarkers in Oral Fluids for Diagnosis of Periodontitis

Dña. Nora Adriana Arias Bujanda

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Accuracy of Molecular Biomarkers in Oral Fluids for Diagnosis of Periodontitis

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"Education is not something you can finish"

Isaac Asimov

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Publications Derived from this Thesis

1. Tomás I, Regueira-Iglesias A, López M, Arias-Bujanda N, Novoa L, Balsa-Castro C, Tomás M. Quantification by qPCR of pathobionts in chronic periodontitis: development of predictive models of disease severity at site-specific level. Front Microbiol. 2017;8: 1443. doi:10.3389/fmicb.2017.01443.

   -Impact factor: 4.076.

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   -Impact factor: 4.164.

   -Ranking 2018: D1, 4/90 (Category: Dentistry, Oral Surgery & Medicine).

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   -Ranking 2018: D1, 4/90 (Category: Dentistry, Oral Surgery & Medicine).

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   -Impact factor: 4.164.

   -Ranking: 2018: D1, 4/90 (Category: Dentistry, Oral Surgery & Medicine).

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RESUMEN DE LA TESIS

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Resumen de la Tesis

“Precisión de los Biomarcadores Moleculares en los Fluidos Orales para el diagnóstico de la Periodontitis”

INTRODUCCIÓN

La periodontitis, la afección más severa de las enfermedades periodontales, está caracterizada por la destrucción de las estructuras de soporte de los dientes [1]. La periodontitis es una enfermedad crónica y multifactorial iniciada por un desequilibrio entre la microbiota subgingival y la homeostasis del huésped [2]. Las enfermedades periodontales (tanto la gingivitis, la forma más leve y reversible de la enfermedad, como la periodontitis) junto con la caries dental, representan las dos enfermedades más frecuentes de la boca [3], y se encuentran entre las enfermedades más prevalentes en todo el mundo [4]. La periodontitis afecta a más del 50% de la población adulta y llega a alcanzar el 11% en sus formas graves [5]. En 2010, la periodontitis se estimó como la sexta enfermedad más prevalente en el mundo [6]. En los Estados Unidos de América (USA), la prevalencia de la periodontitis fue del 47% en adultos mayores de 30 años, lo que equivale a unos 65 millones de personas afectadas [7]. En Europa, por encima de los 60 años, este porcentaje aumenta al 70-85% [8].

La periodontitis tiene múltiples factores de riesgo: locales, sistémicos, congénitos y socioeconómicos [1]. Según la literatura, la periodontitis no es un problema "silente", sino que se ha demostrado que los pacientes periodontales tienen una percepción más pobre de su salud bucal y una peor calidad de vida en comparación con los individuos sanos [9]. Una periodontitis avanzada puede comprometer diferentes funciones de la vida diaria, incluyendo la masticación o incluso el habla, debido a las dificultades en la pronunciación [10-12].

Además, dado que generalmente esta patología conlleva a la pérdida de dientes, la periodontitis se asocia con una peor nutrición, causada por la reducción de la diversidad de alimentos [13,14]. Por último, pero no menos importante, la estética de la sonrisa puede verse afectada en la periodontitis avanzada.

Por otro lado, desde hace años se ha afirmado que la periodontitis está relacionada con graves enfermedades sistémicas como las enfermedades cardiovasculares [15], la diabetes [16] o la artritis reumatoide [17]. En los últimos años también han aparecido numerosas asociaciones epidemiológicas con enfermedades respiratorias [18], complicaciones en el embarazo [19] o incluso el Alzheimer [20].

A pesar de toda la información publicada, existe falta de consenso sobre la definición específica de la periodontitis en la literatura [21]. La mayoría de los autores basan el diagnóstico sólo en criterios clínicos, pero los parámetros utilizados varían según los estudios. El color de las encías, las recesiones gingivales o la profundidad de las bolsas periodontales son algunos de los signos comúnmente estudiados para evaluar las enfermedades periodontales. Otros autores incluyen también la comprobación de la pérdida ósea radiográfica en los criterios diagnósticos.

La clasificación más reciente de las enfermedades periodontales, publicada en 2018, fue realizada por un consenso de expertos de todo el mundo. En esta nueva clasificación, los autores distinguen entre tres formas de periodontitis: "periodontitis necrotizante", "periodontitis como manifestación de enfermedad sistémica" y "periodontitis"[6,22]. La clasificación se caracteriza, por un lado, en diferentes etapas en función de la gravedad y la complejidad del tratamiento de la enfermedad y, por otro, en grados, en función del riesgo de progresión rápida y de los factores individuales de cada paciente, como el hábito de fumar o la hiperglucemia, que se utilizan como modificadores de grado [6,22].

Hoy en día, los parámetros clínicos son el mejor método del que disponemos para diagnosticar y evaluar la severidad de la periodontitis. Sin embargo, los parámetros clínicos sólo son parcialmente capaces de determinar la actividad actual de la enfermedad debido a que algunos de ellos, como la pérdida de inserción clínica (clinical attachment loss, CAL), miden principalmente episodios pasados de destrucción ósea [6,23,24]. Los biomarcadores moleculares se han incluido también en la nueva clasificación de la periodontitis, ya que podrían ser muy útiles en el diagnóstico precoz y monitorización de la periodontitis [6].

Como hemos mencionado, la periodontitis requiere de la interacción de las bacterias subgingivales y la respuesta inmune del huésped para su iniciación y progresión [25]. Según los últimos estudios, la periodontitis crónica se caracteriza por una comunidad bacteriana con mayor diversidad, composición y estructura que la que se encuentra en la salud bucal. Sin embargo, el origen de esta disbiosis es menos conocido. Por un lado, algunos polimorfismos genéticos han sido asociados a un mayor riesgo de periodontitis. Por otro, un segundo factor bien establecido es la acumulación de placa, que podría iniciar una respuesta inflamatoria por parte del huésped susceptible, dando lugar a un entorno bacteriano rico en el fluido gingival crevicular (gingival crevicular fluid, GCF), restos de tejidos y proteínas como las citoquinas, que podrían favorecer a bacterias proteolíticas e "inflamofílicas" como los patógenos periodontales [26]. Esto a su vez aumentaría las proporciones de los patógenos, induciendo una mayor inflamación en un bucle de retroalimentación.

Las citoquinas son proteínas solubles con un papel esencial en la homeostasis, ya que están implicadas en la iniciación y mediación de los procesos inflamatorios [27]. En la fase aguda de la inflamación, las citoquinas son liberadas por células epiteliales, fibroblastos y fagocitos; mientras que en la inmunidad adquirida, son liberadas por linfocitos [28,29]. Su producción en el organismo está extremadamente regulada, y por ello, concentraciones más altas de citoquinas se asocian con inflamación y progresión de la enfermedad [30]. Además, las citoquinas actúan como una red por lo que diferentes citoquinas realizan las mismas funciones, permitiendo que en ausencia de una citoquina

específica otra con una actividad similar pueda ocupar su lugar, activándose así la respuesta por otra vía [31].

La toma de muestras en el sitio exacto de la enfermedad proporciona una gran cantidad de información, y debido al hecho de que las citoquinas se producen localmente en los tejidos periodontales, el análisis de los niveles de citoquinas en el GCF se considera un medio preciso para diagnosticar los niveles locales de inflamación [32]. La importancia del GCF radica también en que es un "elemento intermediario" entre la placa bacteriana adherida al diente y los tejidos periodontales [33,34].

La saliva, por otro lado, es considerada como el "espejo del cuerpo". Se considera un fluido atractivo para el diagnóstico de la periodontitis ya que no sólo es fácil y no invasiva su forma de recoger, sino que además permite acumular abundante cantidad [24,35]. La composición de la saliva es una mezcla de las glándulas salivales, GCF, suero, secreciones expectoradas, bacterias y diferentes tipos de células humanas [36]. Aunque hay pruebas que sugieren que la principal fuente de citoquinas en la saliva es el GCF, la dilución de este fluido que contiene estas citoquinas en la saliva puede explicar la falta de consenso en la literatura. Algunos estudios sugieren que los biomarcadores salivales podrían discriminar entre salud y enfermedad [37-39] mientras que otros no encuentran diferencias [40,41]. La metaloproteinasa de matriz (matrix metalloproteinase, MMP) 8 ha sido considerada como un prometedor biomarcador de la periodontitis [42].

Existen diferentes técnicas para medir estos biomarcadores en la periodontitis, aunque normalmente se eligen los inmunoensayos, ya que son específicos para los biomarcadores, fáciles de utilizar, precisos y estandarizados [43]. Durante años, el ensayo inmunoenzimático (enzyme-linked immunosorbent assay, ELISA), fue el estándar para el análisis cuantitativo de citoquinas y otros biomarcadores inflamatorios [44]. Desde entonces, los ensayos de citoquinas han ido evolucionando y la aparición de nuevas técnicas como la citometría multiparamétrica ha revolucionado la biología experimental de manera que nos permite

detectar y cuantificar múltiples citoquinas en una sola muestra y en un pequeño volumen [30,45].

Sin embargo, aunque en la literatura hay muchos trabajos que analizan y comparan diferentes perfiles de citoquinas y otras moléculas en pacientes sanos y periodontales, sólo unos pocos estudios se centran en el análisis de la fiabilidad diagnóstica [38,46-49].

JUSTIFICACIÓN Y OBJETIVOS

La existencia de un perfil de biomarcador asociado a la periodontitis en los fluidos orales no indica su capacidad diagnóstica. Las investigaciones sobre fiabilidad diagnóstica requieren el diseño de un estudio específico que proporciona medidas de clasificación del biomarcador (por ejemplo, sensibilidad y especificidad) [50].

Los profesionales de la salud que buscan evidencia sobre pruebas diagnósticas tienden a recurrir a revisiones sistemáticas sobre precisión de las pruebas diagnósticas [51]. A pesar de ser un tema de gran interés para la comunidad científica, no existen revisiones sistemáticas/metaanálisis sobre fiabilidad diagnóstica que revelen qué moléculas detectadas en los fluidos orales son los biomarcadores más prometedores para el diagnóstico de la periodontitis.

Por otro lado, existe escasa evidencia en la literatura sobre el desarrollo y validación de modelos predictivos basados en citoquinas orales para el diagnóstico de la periodontitis mediante técnicas apropiadas de modelización predictiva [52]. Además, aún no se ha evaluado la influencia del tabaquismo en la capacidad diagnóstica de estas moléculas en los fluidos orales.

En consecuencia, la falta de evidencia sobre la precisión de los biomarcadores moleculares en los fluidos orales para el diagnóstico de la periodontitis nos motivó a iniciar y desarrollar esta área de investigación. Por ello, en esta Tesis Doctoral proponemos los siguientes objetivos:

1) Analizar, mediante un enfoque metaanalítico, la precisión diagnóstica de los biomarcadores moleculares en el GCF para la detección de la periodontitis en sujetos sistémicamente sanos.

2) Analizar, mediante un enfoque metaanalítico, la precisión diagnóstica de los biomarcadores moleculares en saliva para la detección de la periodontitis en sujetos sistémicamente sanos.

3) Obtener modelos predictivos basados en citoquinas en el GCF que permitan distinguir a los sujetos sistémicamente sanos con periodontitis de aquellos con salud periodontal, desarrollando sus correspondientes nomogramas de aplicación clínica y describiendo sus medidas aparentes y corregidas de discriminación y clasificación.

4) Determinar los umbrales diagnósticos de periodontitis derivados de los mejores modelos basados en citoquinas en el GCF y en ratios de citoquinas en pacientes sistémicamente sanos, tanto no fumadores como fumadores, describiendo medidas aparentes y corregidas de discriminación y clasificación.

5) Obtener modelos predictivos basados en interleuquina (interleukin, IL) 1beta en saliva que permitan diferenciar entre pacientes sistémicamente sanos con periodontitis no tratada de aquellos con salud periodontal y con periodontitis tratada, diferenciando entre fumadores y no fumadores, desarrollando sus correspondientes nomogramas de aplicación clínica y describiendo los diferentes umbrales de diagnóstico y las medidas de rendimiento.

OBJETIVO 1. PRECISIÓN DE LOS BIOMARCADORES MOLECULARES INDIVIDUALES EN EL FLUIDO GINGIVAL CREVICULAR PARA EL DIAGNÓSTICO DE LA PERIODONTITIS: REVISIÓN SISTEMÁTICA Y METAANÁLISIS

1.1. MATERIAL Y MÉTODOS

Se planteó la realización de una búsqueda sistemática de la literatura en seis bases de datos electrónicas: Pubmed (Medline), Embase, el Registro Cochrane Central de Ensayos Controlados (Cochrane Central Register of Controlled Trials and Trial Protocols), Scopus, Lilacs y Web of Sciences (WoS). Para ello se elaboró un protocolo siguiendo las directrices del Grupo Cochrane para revisiones sistemáticas de precisión de pruebas diagnósticas [53] y la declaración PRISMA [50] y se planteó la siguiente pregunta PICO (patient, index test, comparison, outcome): "En sujetos sistémicamente sanos, ¿la expresión de biomarcadores moleculares individuales en el GCF muestra capacidad diagnóstica de periodontitis en comparación con los parámetros clínicos convencionales?".

Se incluyeron aquellos estudios (transversales, longitudinales o intervencionistas) sobre biomarcadores moleculares en el GCF que proporcionaron resultados sobre precisión diagnóstica en individuos con periodontitis clínicamente diagnosticada (estándar de referencia). Se excluyeron los estudios sin tablas de contingencia para la clasificación binaria (tablas 2x2) o sin valores de sensibilidad y especificidad ni tamaños muestrales de los pacientes incluidos a partir de las cuales fuera posible calcular las tablas de clasificación. Los estudios de precisión pronóstica y predictiva también fueron excluidos.

Los participantes incluidos fueron pacientes de cualquier edad sin un diagnóstico explícito de enfermedad sistémica y con un diagnóstico periodontal clínico. Se excluyeron los estudios sobre pacientes con afecciones sistémicas, experimentación animal o modelos *in vitro*.

El estándar de referencia para el diagnóstico de la periodontitis se basó únicamente en parámetros clínicos (profundidad de la bolsa periodontal -probing pocket deph, PPD- o CAL) o en parámetros

clínicos y radiográficos (pérdida ósea -bone loss, BL-). Cualquier biomarcador molecular único detectado en el GCF analizado desde un punto de vista de la precisión diagnóstica, se consideró un biomarcador diagnóstico.

La estrategia de búsqueda utilizada en las diferentes bases de datos electrónicas se realizó el 25 de octubre de 2018 y se detalla en la Tabla 1 del Objetivo 1. Aplicando la estrategia de búsqueda se realizaron un total de 176 búsquedas en cada base de datos. Los resúmenes de todos los artículos se analizaron computacionalmente, aunque se analizaron manualmente los artículos con identificadores múltiples publicados (PubMed identifier, PMID), aquellos con un solo PMID que no proporcionaron un resumen o aquellos que no tenían un PMID designado. La selección manual fue realizada por dos revisores independientes (NAB y ARI). Se definieron una serie de palabras positivas y negativas, tanto en singular como en plural: las palabras relacionadas con aspectos de los modelos predictivos y las pruebas de clasificación binaria se consideraron positivas mientras que las asociadas con los modelos de experimentación animal se consideraron negativas.

Tras la selección de títulos y resúmenes, los estudios encontrados por el proceso automatizado y los detectados por ambos revisores se fusionaron en una única base de datos, que incluía los textos completos de los artículos candidatos. El análisis de los textos completos fue realizado por dos revisores independientes (NAB y ARI). Se registraron los motivos de exclusión de los estudios y la calidad de los estudios incluidos se comprobó mediante la lista de verificación revisada "*quality assessment of diagnostic studies*" (QUADAS-2) [54]. Cuatro autores (NAB, ARI, IT y CBC) extrajeron independientemente los datos por duplicado usando un formulario estandarizado. Los dos primeros autores se centraron en las características de los estudios, mientras que los dos segundos se centraron en los datos de precisión diagnóstica de los biomarcadores.

Como parámetros de clasificación, se expresaron los valores de sensibilidad y especificidad, y sus correspondientes intervalos de

confianza (confidence intervals, CIs) al 95%, para cada clasificación de un biomarcador en GCF, así como también se determinaron otras medidas del rendimiento del biomarcador.

El metaanálisis se realizó cuando el número de clasificaciones diagnósticas de un biomarcador en el GCF fue de al menos tres, obtenidas de al menos tres artículos. Para realizar el metaanálisis se utilizó el modelo jerárquico o modelo HSROC (hierarchical summary receiver operating characteristic). Con el objetivo de tratar de aportar evidencia directa sobre las consecuencias clínicas de los biomarcadores moleculares en GCF sometidos a análisis metaanalítico [51], se calcularon los valores predictivos porcentuales sobre una cohorte hipotética de 1000 pacientes, teniendo en cuenta las estimaciones de sensibilidad, especificidad y las diferentes prevalencias de la periodontitis [55].

1.2. Resultados

En total se obtuvieron 8.410 artículos de las seis bases de datos. De éstos, el 87,3% de los resúmenes se estudiaron mediante técnicas automatizadas de extracción de datos y el 12,7% restante mediante un procedimiento manual. Después de seleccionar los artículos con al menos una palabra positiva y ninguna negativa, se evaluaron 120 artículos en lectura completa. Además, se detectaron siete artículos más después de estudiar las referencias de una lista de revisiones y de los artículos de texto completo.

Finalmente, se excluyeron 108 artículos por diversas razones (Apéndice S2 del Objetivo 1), y se seleccionaron 19 artículos y 69 clasificaciones binarias para el análisis cualitativo. Tras aplicar los requisitos establecidos para el metaanálisis, se seleccionaron nueve artículos y 24 clasificaciones binarias para el análisis cuantitativo.

En 8/19 trabajos (42,1%), los autores investigaron la precisión diagnóstica de un solo biomarcador en el GCF, mientras que en los restantes 11/19 (57,9%) se compararon al menos dos biomarcadores. Se identificaron un total de 36 biomarcadores moleculares individuales, de los cuales: 20 (55,6%) fueron enzimas; ocho (22,2%) fueron mediadores inflamatorios y de respuesta del huésped; cinco (13,9%) fueron productos relacionados con la degradación de los tejidos periodontales; y tres (8,3%) fueron clasificados como "otros". Veintiuno de los 36 biomarcadores (58,3%) sólo fueron evaluados en un artículo. Las técnicas más utilizadas fueron los métodos colorimétricos o fluorimétricos, seguidos de la citometría multiparamétrica y la técnica ELISA.

Sólo cuatro de los 36 biomarcadores tuvieron al menos tres clasificaciones diagnósticas en al menos tres artículos, y por lo tanto, fueron seleccionados para los meta-análisis. Estas cuatro moléculas fueron todas enzimas: MMP8, elastasa, catepsina y tripsina. La sensibilidad y especificidad estimadas fueron: para MMP8, 76,7% y 92,0%; para la elastasa, 74,6% y 81,1%; para la catepsina, 72,8% y 67,3% respectivamente. Las peores estimaciones de sensibilidad y especificidad fueron para tripsina (71,3% y 66,1%, respectivamente).

En términos de utilidad o eficacia clínica de los dos biomarcadores en GCF más estudiados (MMP8 y elastasa), y considerando una prevalencia del 45% de periodontitis [56,57], el 88,8% del total de pruebas positivas del MMP8 indicaría un verdadero positivo; mientras que del total de pruebas negativas del MMP8, el 82,8% correspondería a un verdadero negativo. Para una prueba de elastasa, estos porcentajes serían 79,7% y 76,4%, respectivamente.

Respecto al análisis cualitativo, si nos centramos en aquellas clasificaciones diagnósticas que presentaron mejores de precisión (accuracy, ACC) superiores al 90%, para el diagnóstico de la periodontitis (valores de sensibilidad/especificidad), Baeza et al. [46] obtuvieron un ACC del 95,2% (93,5%/96,8%) para ProMMP2 y un valor del 95,2% (96,8%/93,5%) para ProMMP9. Para otras enzimas, Leppilahti et al. [58] detectaron un ACC de 94,7% (94,7%/94,7%) y

91,4% (94,7%/89,7%) para la mieloperoxidasa (myeloperoxidase, MPO) y MMP14, respectivamente. En cuanto a los biomarcadores inflamatorios, Tomás et al. [59] observaron que la IL1beta presentó un ACC del 93,9% (93,2%/94,6%), mientras que IL1alpha, un ACC del 93,2% (94,5%/91,9%).

Objetivo 2. ¿Qué Precisión Tienen los Biomarcadores Moleculares Individuales en la Saliva para el Diagnóstico de la Periodontitis? Revisión Sistemática y Metaanálisis

2.1. Material y Métodos

Se planteó la realización de una búsqueda sistemática en la literatura mediante seis bases de datos electrónicas: Pubmed (Medline), Embase, el Registro Cochrane Central de Ensayos Controlados (Cochrane Central Register of Controlled Trials and Trial Protocols), Scopus, Lilacs y Web of Sciences (WoS). Para ello se efectuó un protocolo siguiendo las directrices del Grupo Cochrane para revisiones sistemáticas de precisión de pruebas diagnósticas [53] y la declaración PRISMA [50] y se planteó la siguiente pregunta PICO: "En sujetos sistémicamente sanos, ¿la expresión de biomarcadores moleculares individuales en saliva muestra capacidad diagnóstica de periodontitis en comparación con los parámetros clínicos convencionales?".

Se incluyeron aquellos estudios (transversales, longitudinales o intervencionistas) sobre biomarcadores moleculares en saliva que proporcionaron resultados sobre fiabilidad diagnóstica en individuos con periodontitis clínicamente diagnosticada (estándar de referencia).

Se excluyeron los estudios sin tablas de contingencia para la clasificación binaria (tablas 2x2) o sin valores de sensibilidad y especificidad ni tamaños muestrales de los pacientes incluidos a partir de las cuales fuera posible calcular las tablas de clasificación. Los estudios de precisión pronóstica y predictiva también fueron excluidos.

Los participantes incluidos fueron pacientes de cualquier edad sin un diagnóstico explícito de enfermedad sistémica y con un diagnóstico periodontal clínico. Se excluyeron los estudios sobre pacientes con afecciones sistémicas, experimentación animal o modelos *in vitro*.

El estándar de referencia para el diagnóstico de la periodontitis se basó únicamente en parámetros clínicos (PPD o CAL) o en parámetros clínicos y radiográficos (BL). Cualquier biomarcador molecular único detectado en saliva analizado desde un punto de vista de la precisión diagnóstica, se consideró un biomarcador diagnóstico.

La estrategia de búsqueda utilizada en las diferentes bases de datos electrónicas se realizó el 25 de octubre de 2018 y se detalla en la Tabla 1 del Objetivo 2. Aplicando la estrategia de búsqueda se realizaron un total de 176 búsquedas en cada base de datos. Los resúmenes de todos los artículos se analizaron computacionalmente, aunque se analizaron manualmente los artículos con PMID múltiples publicados, aquellos con un solo PMID que no proporcionaron un resumen o aquellos que no tenían un PMID designado. La selección manual fue realizada por dos revisores independientes (NAB y ARI). Se definieron una serie de palabras positivas y negativas, tanto en singular como en plural: las palabras relacionadas con aspectos de los modelos predictivos y las pruebas de clasificación binaria se consideraron positivas mientras que las asociadas con los modelos de experimentación animal se consideraron negativas.

Tras la selección de títulos y resúmenes, los estudios encontrados por el proceso automatizado y los detectados por ambos revisores se fusionaron en una única base de datos, que incluía los textos completos de los artículos candidatos. El análisis de los textos completos fue realizado por dos revisores independientes (NAB y ARI). Se registraron los motivos de exclusión de los estudios y la calidad de los estudios incluidos se comprobó mediante la herramienta QUADAS-2 [54]. Cuatro autores (NAB, ARI, IT y CBC) extrajeron independientemente los datos por duplicado usando un formulario estandarizado. Los dos primeros autores se centraron en las características de los estudios,

mientras que los dos segundos se centraron en los datos de precisión diagnóstica de los biomarcadores.

Como parámetros de clasificación, se expresaron los valores de sensibilidad y especificidad, y sus correspondientes CIs al 95%, para cada clasificación de un biomarcador en saliva, así como también se determinaron otras medidas del rendimiento del biomarcador.

El metaanálisis se realizó cuando el número de clasificaciones diagnósticas de un biomarcador en saliva fue de al menos tres, obtenidas de al menos tres artículos. Para realizar el metaanálisis se utilizó el modelo HSROC. Con el objetivo de tratar de aportar evidencia directa sobre las consecuencias clínicas de los biomarcadores en saliva sometidos a análisis metaanalítico [51], se calcularon los valores predictivos porcentuales sobre una cohorte hipotética de 1000 pacientes, teniendo en cuenta las estimaciones de sensibilidad, especificidad y las diferentes prevalencias de la periodontitis [55].

2.2. Resultados

En total, tras la eliminación de duplicados, se obtuvieron 4.511 artículos de las seis bases de datos. El 90,5% de los resúmenes se evaluó mediante técnicas automatizadas de extracción de datos y el 9,5% restante mediante un procedimiento manual. Un total de 104 artículos fueron seleccionados para su lectura completa. Además, se detectaron seis artículos más después de analizar las referencias de una lista de revisiones y los artículos de texto completo. Finalmente, se excluyeron 92 artículos por diversas razones (Apéndice S2 del Objetivo 2), con lo cual se evaluaron 18 publicaciones (86 clasificaciones binarias) en el análisis cualitativo. Tras aplicar los requisitos establecidos para los metaanálisis, se consideraron 12 artículos (36 clasificaciones binarias) para el análisis cuantitativo.

En 8/18 artículos (44,4%) solo se evaluó un único biomarcador, mientras que en los 10/18 restantes (55,6%) se evaluó más de uno. Se identificaron un total de 32 biomarcadores moleculares individuales, de los cuales 13 (40,6%) fueron mediadores inflamatorios y de respuesta del huésped, nueve (28,1%) fueron enzimas, seis (18,8%) fueron

productos relacionados con la degradación de los tejidos periodontales y cuatro (12,5%) se clasificaron como "otros".

En cuanto al tipo de saliva analizada, predominó la saliva no estimulada (63,2%), seguida de la saliva estimulada (21,0%). Las técnicas más utilizadas para la identificación de biomarcadores salivales fueron la técnica ELISA (54,4%) y la citometría multiparamétrica (17,6%).

De los 32 biomarcadores, sólo cinco obtuvieron al menos tres clasificaciones en al menos tres artículos, y por lo tanto fueron seleccionados para los metaanálisis. Los mejores valores de sensibilidad se obtuvieron para IL1beta (78,7%), seguido de MMP8 (72,5%), IL6 y hemoglobina (haemoglobin -Hb-; 72,0% para ambas moléculas); los peores valores de sensibilidad fueron para MMP9 (70,3%). En cambio, MMP9 tuvo los mejores resultados de especificidad (81,5%), seguido de IL1beta (78,0%) y Hb (75,2%); MMP8 se asoció con los peores valores de especificidad (70,5%).

En relación a la utilidad o efectividad de los dos biomarcadores más estudiados en saliva (MMP8 e IL1beta), considerando los estimadores de sensibilidad y especificidad obtenidos a partir del meta-análisis y una prevalencia del 45% de periodontitis [56,57], el 67,2% del total de pruebas positivas de IL1beta indicaría un verdadero positivo; mientras que del total de pruebas negativas de IL1beta, el 78,7% correspondería a un verdadero negativo. Para una prueba MMP8, estos porcentajes serían 63,4% y 75,2%, respectivamente.

Respecto al análisis cualitativo, si nos centramos en aquellos biomarcadores salivales que mostraron mejores valores de ACC, superiores al 90%, para el diagnóstico de la periodontitis (valores de sensibilidad/especificidad), Tassi y Lotito [60] obtuvieron un ACC del 97,0% (97,3%/96,8%) para la cisteína, Al-Sabbagh et al. [61] un ACC del 93,8% (95,0%/92,5%) para la proteína inflamatoria de macrófagos (macrophage inflammatory protein, MIP) 1alfa, y Bejeh-Mir et al. [62] un ACC del 95,2% (92,9%/96,4%) para el óxido nítrico, nitrato y nitrito.

Objetivo 3. Modelos Predictivos Basados en Citoquinas para Estimar la Probabilidad de Periodontitis Crónica: Desarrollo de Nomogramas Diagnósticos

3.1. Material y Métodos

Se seleccionaron 150 participantes, 75 controles periodontalmente sanos (grupo control) y 75 sujetos con periodontitis crónica generalizada moderada-severa (grupo perio). Los pacientes seleccionados cumplieron los siguientes criterios de inclusión: edad entre 30 y 75 años; no presencia de enfermedades sistémicas; sin antecedentes de abuso de alcohol o drogas; no presencia de embarazo o lactancia materna; no ingesta de antibióticos durante los últimos seis meses o antiinflamatorios en los últimos cuatro; no uso habitual de antisépticos orales; no presencia de implantes o aparatos de ortodoncia; no antecedentes de tratamiento periodontal previo; fumadores durante un período mínimo de ocho años; no haber fumado nunca o fumadores que hubieran dejado el hábito al menos cinco años antes de la toma de muestras; y presencia de al menos 18 dientes naturales.

Un dentista experimentado previamente calibrado realizó todos los diagnósticos periodontales. Los parámetros PPD y CAL se registraron en toda la boca en seis sitios por diente. Se registró también el sangrado al sondaje (bleeding on probing, BOP) y el nivel de placa bacteriana (bacterial plaque levels, BPL) en toda la boca. Se obtuvieron radiografías periapicales de todos los dientes para evaluar el estado del hueso alveolar.

La presencia o ausencia de periodontitis crónica se basó en información clínica/radiográfica. El grupo control incluyó individuos periodontalmente sanos con BOP <25%, ninguna localización con PPD ≥4 mm y sin evidencia radiográfica de pérdida ósea alveolar. Los pacientes del grupo perio fueron diagnosticados de periodontitis crónica generalizada moderada-severa en base a los criterios previamente establecidos [63,64]. La información sobre el hábito tabáquico se obtuvo mediante la aplicación de un cuestionario (ítems recogidos: no fumador, exfumador, fumador actual, tiempo pasado como exfumador o como fumador, y número de cigarrillos consumidos al día).

La obtención del GCF tuvo lugar una semana después del examen inicial, y las muestras se obtuvieron a la misma hora del día (por la tarde, aproximadamente 5-7 horas tras el cepillado). Se insertó una tira de papel (Periopaper, Amityville, NY, USA) en el surco gingival o bolsa periodontal durante 30 segundos. Las muestras de GCF de los pacientes periodontalmente sanos se tomaron de 20 localizaciones no adyacentes entre sí y se agruparon en un único tubo por sujeto. En los pacientes periodontales, las 20 muestras subgingivales fueron extraídas de las localizaciones con mayor valor de PPD en cada cuadrante y posteriormente agrupadas en un único tubo por sujeto.

Un solo investigador realizó los análisis cuantitativos de citoquinas. Los niveles de citoquinas en el GCF fueron determinados mediante un inmunoensayo multiparamétrico (Affymetrix, Inc., Santa Clara, CA, USA) y el instrumento Luminex 100™ (Luminex Corporation, Austin, Texas, USA). Se midieron 16 mediadores: 1) ocho citoquinas proinflamatorias (factor estimulante de colonias de granulocitos y macrófagos -granulocyte-macrophage colony-stimulating factor, GMCSF-, IL1alfa, IL1beta, IL6, IL12p40, IL17A, IL17F y factor de necrosis tumoral -tumour necrosis factor, TNF- alfa); y 2) ocho citoquinas antiinflamatorias (interferón -interferon, IFN-gamma, IL2, IL3, IL4, IL5, IL10, IL12p70 e IL13).

Los análisis estadísticos se realizaron utilizando el software R. Se utilizó la prueba de U de Mann-Whitney para comparar las características clínicas cuantitativas entre ambos grupos y el test exacto de Fisher para evaluar la asociación entre las variables cualitativas. El nivel de significación aplicado fue $p<0,05$.

Debido a la distribución anormal de las citoquinas, los valores fueron transformados logarítmicamente (log2) para los análisis estadísticos. Se calcularon las correlaciones de Spearman entre las citoquinas y se utilizaron como orientación para la construcción del modelo, con el fin de evitar redundancias y una posible colinealidad entre citoquinas con efectos biológicos similares. Los modelos basados en citoquinas se seleccionaron por su importancia biológica, su capacidad para predecir la periodontitis crónica y su validez estadística.

Se seleccionaron aquellos modelos que presentaron valores más altos de área bajo la curva (area under the curve, AUC). Cabe señalar que los modelos con un valor AUC igual o superior a 0,70 se consideran modelos predictivos aceptables [65].

El test de Hosmer-Lemeshow se aplicó a los modelos seleccionados [66]. Los nomogramas se construyeron a partir de los resultados de los análisis multivariables [67]. Un nomograma mapea las probabilidades pronosticadas en puntos en una escala de 0 a 100 en una interfaz gráfica fácil de usar. Los puntos totales acumulados por las distintas covariables corresponden a la probabilidad de enfermedad prevista para un paciente [68].

Los modelos se verificaron internamente mediante métodos "*bootstrap*" para verificar el posible sobreajuste. Se determinaron los valores del optimismo sobre las medidas aparentes de discriminación, clasificación y calibración, para posteriormente, obtener las medidas corregidas (bias-corrected, bc) de discriminación, clasificación y calibración [69,70].

3.2. RESULTADOS

Los niveles de todas las citoquinas proinflamatorias fueron significativamente más altos en el grupo perio en comparación con el grupo control (p<0,001, para todas las comparaciones). En cuanto a las citoquinas antiinflamatorias, sólo cuatro mediadores (IFNgamma, IL2, IL3 e IL4) presentaron concentraciones significativamente mayores en el grupo perio (p<0,001, para todas las comparaciones). El aumento de las concentraciones de estas citoquinas antiinflamatorias fue menor que el detectado para las citoquinas proinflamatorias, excepto para la IL3.

Casi todas las correlaciones entre las citoquinas, tanto proinflamatorias como antiinflamatorias, fueron positivas. Se detectaron fuertes correlaciones positivas entre algunas citoquinas proinflamatorias, en concreto IL1alfa, IL1beta e IL17A (rho>0,85).

Respecto a los modelos de una sola citoquina ajustados por la covariable "fumar", las citoquinas proinflamatorias IL1alfa, IL1beta e

IL17 fueron los predictores que mostraron valores más altos de AUC (0,973, 0,963, 0,937, respectivamente). En cuanto a los modelos de dos citoquinas ajustados por "fumar", la incorporación de ciertas citoquinas antiinflamatorias mejoró los valores de AUC de los mejores modelos basados en una sola citoquina proinflamatoria, especialmente el de IL17A (de 0,937 a 0,974). Estos modelos de dos citoquinas fueron: IL1alfa + IFNgamma, IL1beta + IL10 e IL17A + IFNgamma.

Los nomogramas diagnósticos derivados de los seis modelos basados en citoquinas obtuvieron una extraordinaria precisión, debido a los altos valores de discriminación y medidas de clasificación. De acuerdo con los parámetros de calibración, especialmente el nomograma basado en la IL1alfa y el de la IL17 + IFNgamma fueron muy fiables, ya que mostraron muy buena correspondencia entre los resultados reales y las probabilidades pronosticadas de tener periodontitis crónica. En los tres nomogramas de dos citoquinas, los niveles más altos de citoquinas proinflamatorias se asociaron con una mayor probabilidad de tener periodontitis crónica, al igual que ser fumador. Por el contrario, IFNgamma e IL10 tuvieron una función opuesta a las proinflamatorias, ya que los niveles más altos de estos mediadores se asociaron con una menor probabilidad de tener periodontitis.

OBJETIVO 4. UMBRALES DE CITOQUINAS EN EL FLUIDO GINGIVAL CREVICULAR CON POTENCIAL PIAGNÓSTICO DE PERIODONTITIS CRÓNICA DIFERENCIANDO POR EL HÁBITO TABÁQUICO

4.1. MATERIAL Y MÉTODOS

Se seleccionaron 150 participantes, 75 controles periodontalmente sanos (grupo control) y 75 sujetos con periodontitis crónica generalizada moderada-severa (grupo perio). Los pacientes seleccionados cumplieron los criterios de inclusión descritos en el Objetivo 3, ya que se trató de la misma serie de pacientes.

Un dentista experimentado previamente calibrado realizó todos los diagnósticos periodontales. La presencia o ausencia de periodontitis

crónica se basó en información clínica/radiográfica. Los detalles del diagnóstico clínico realizado están descritos en el Objetivo 3.

La obtención del GCF tuvo lugar una semana después del examen inicial, y las muestras se obtuvieron a la misma hora del día. Se insertó una tira de papel (Periopaper, Amityville, NY, USA) en el surco gingival o bolsa periodontal durante 30 segundos. Las muestras de GCF de los pacientes periodontalmente sanos se tomaron de 20 sitios diferentes y se agruparon en un único tubo por sujeto. En los pacientes periodontales, las 20 muestras subgingivales fueron extraídas de las localizaciones con mayor valor de PPD en cada cuadrante y posteriormente agrupadas en un único tubo por sujeto.

Un solo investigador realizó los análisis cuantitativos de citoquinas. Los niveles de citoquinas en el GCF fueron determinados usando mediante un inmunoensayo multiparamétrico (Affymetrix, Inc., Santa Clara, CA, USA) y el instrumento Luminex 100™ (Luminex Corporation, Austin, Texas, USA). Se midieron 16 mediadores: 1) ocho citoquinas proinflamatorias (GMCSF, IL1alfa, IL1beta, IL6, IL12p40, IL17A, IL17F y TNFalfa); y 2) ocho citoquinas antiinflamatorias (IFNgamma, IL2, IL3, IL4, IL5, IL10, IL12p70 e IL13).

Los análisis estadísticos se realizaron utilizando el software R. Debido a la distribución anormal de las variables, se utilizó la prueba de U de Mann-Whitney para comparar los niveles de citoquinas y las ratios de citoquinas (cociente entre dos citoquinas) entre los grupos control y perio. Los niveles de significación aplicados se ajustaron mediante la corrección Benjamini-Hochberg [71]. Se evaluaron 66 ratios de citoquinas, teniendo en cuenta sólo aquellas citoquinas que mostraron niveles significativos en los pacientes periodontales en comparación con los controles.

Para obtener los umbrales de diagnóstico específicos diferenciados por el hábito tabáquico, se decidió desarrollar modelos predictivos diferentes para no fumadores y fumadores (n=93 y 54, respectivamente). Los modelos se construyeron seleccionando una citoquina individual o una ratio de citoquinas como variable predictiva.

El criterio estadístico aplicado para la selección de modelos fue la capacidad de cada modelo basado en citoquinas individuales o basado en ratios de citoquinas para discriminar la presencia de periodontitis crónica utilizando el valor del AUC [72]. Sólo aquellos modelos que presentaron un AUC aparente ≥0,85 en ambos tipos de modelos para fumadores y no fumadores fueron seleccionados [65].

El umbral de clasificación óptimo para cada modelo se definió como aquel que proporciona el porcentaje máximo de predicciones correctas (máximo ACC). Al establecer este valor óptimo, se obtuvieron varias medidas de clasificación. Las curvas de los modelos seleccionados se construyeron gráficamente [73].

Los modelos se verificaron internamente mediante la técnica de "*bootstrapping*", corrigiendo las medidas aparentes de discriminación y clasificación según los valores del optimismo. Esta técnica también se utilizó para definir los umbrales de citoquinas para los valores medios del ACC derivados de 10.000 muestras de cada modelo seleccionado, así como los umbrales para los CIs al 90% de los valores del ACC.

4.2. RESULTADOS

Todas las citoquinas proinflamatorias, y cuatro citoquinas antiinflamatorias analizadas (IFNgamma, IL2, IL3 e IL4), obtuvieron niveles significativamente más altos en el grupo perio que en el grupo de control (valor *p* ajustado $\leq 1 \times 10^{-3}$). En relación a las ratios de citoquinas, 19 mostraron diferencias significativas entre el grupo control y el grupo perio (valor *p* ajustado $< 1 \times 10^{-5}$).

Tres modelos basados en citoquinas individuales y tres modelos basados en ratios de citoquinas obtuvieron un AUC aparente ≥0,85 tanto para no fumadores como para fumadores. Los modelos fueron IL1alfa, IL1beta, IL17A, IL1alfa/IL2, IL1beta/IL2 e IL17A/IL2.

Los modelos basados en citoquinas individuales obtuvieron valores AUC y bc-AUC ≥0,940 y ≥0,912, respectivamente. Los valores de los modelos basados en ratios de citoquinas fueron ≥0,857 y ≥0,834,

respectivamente. Los rangos bc-ACC derivados de los modelos basados en citoquinas individuales fue del 86,8%-94,1% y los de los modelos basados en ratios de citoquinas fue del 72,9%-88,7%, siendo IL17A e IL17A/IL2 los biomarcadores con los valores más bajos de bc-ACC tanto en no fumadores como en fumadores.

Los umbrales de citoquinas en pg/ml para los valores medios de ACC (CIs al 90% de los valores de ACC) para fumadores y no fumadores fueron, respectivamente: modelo IL1alfa: 46099 (37495-64161) y 65644 (51310-76700); modelo IL1beta: 4732 (3705-6459) y 5827 (4721-7532); modelo IL17A: 11.03 (7.28-15.22) y 17.13 (13.10-22.53); modelo IL1alpha/IL2: 4210 (3164-5648) y 7118 (4798-10166); modelo IL1beta/IL2: 260 (63-487) y 628 (348-897); modelo IL17A/IL2: 0,810 (0,707-1,132) y 1,919 (1,073-3,489). En comparación con los no fumadores, los fumadores tenían umbrales de diagnóstico más bajos en todos los modelos predictivos tanto para los valores ACC aparentes como para los valores ACC obtenidos por bootstrapping.

Objetivo 5. Precisión Diagnóstica de la IL1beta en Saliva: Desarrollo de Modelos Predictivos para Estimar la Probabilidad de Periodontitis en No Fumadores y Fumadores

5.1. Material y Métodos

Se reclutaron 141 participantes, incluyendo 62 sujetos periodontalmente sanos (grupo control) y 79 pacientes afectados por periodontitis sin tratar (grupo de periodontitis no tratada). Los pacientes seleccionados cumplieron los siguientes criterios de inclusión: edad entre 30 y 75 años; no presencia de enfermedades sistémicas; sin antecedentes de abuso de alcohol o drogas; no presencia de embarazo o lactancia materna; no ingesta de antibióticos durante los últimos seis meses o antiinflamatorios en los últimos cuatro; no uso habitual de antisépticos orales; no presencia de implantes o aparatos de ortodoncia; no antecedentes de tratamiento periodontal previo; haber fumado durante al menos un año; no haber fumado nunca o haber dejado de fumar hace más de 3 años; y presencia de al menos 18 dientes naturales.

Dos dentistas experimentados, previamente calibrados, realizaron todos los diagnósticos periodontales. El diagnóstico de la periodontitis se basó en la información clínica y radiográfica obtenida. El grupo control incluyó pacientes con salud periodontal que presentaron BOP <25%, sin localizaciones con PPD ≥4 mm o evidencia radiográfica de pérdida ósea alveolar. El grupo perio no tratado incluyó pacientes diagnosticados con periodontitis (estadios II-IV) aplicando los nuevos criterios de clasificación recientemente publicados [6,22]. El hábito tabáquico se evaluó mediante cuestionario (ítems registrados: no fumador, exfumador, fumador actual, tiempo pasado como exfumador o como fumador, y número de cigarrillos consumidos al día). De los 79 pacientes del grupo perio sin tratar, 60 recibieron tratamiento periodontal básico (raspado y alisado radicular) y después de dos meses fueron reevaluados clínicamente (grupo perio tratado).

Se tomaron muestras de saliva no estimulada de cada paciente usando el "*spitting method*" [74] durante los primeros 10 días tras la exploración intraoral y dos meses después de recibir el tratamiento periodontal básico. Los pacientes evitaron cepillarse los dientes o cualquier otra forma de higiene oral, comer y beber desde una hora antes de la recogida de las muestras.

En el Objetivo 3 demostramos que la IL1beta es un biomarcador excelente para distinguir a los pacientes sistémicamente sanos con periodontitis crónica de los individuos periodontalmente sanos. Sin embargo, como se refleja en el Objetivo 2, se han publicado muy pocos estudios sobre la precisión diagnóstica de la IL1beta en la saliva, y ninguno de ellos ha evaluado el impacto del "hábito de fumar" de un paciente sobre esta capacidad diagnóstica [38,48,49,75-78]. Consecuentemente, un solo investigador ciego a los datos clínicos realizó los análisis cuantitativos de este biomarcador. Los niveles salivales de IL1beta se determinaron utilizando un kit de ultrasensibilidad Milliplex® (Merck Chemicals and Life Science, S.A., Madrid, España) y el instrumento Luminex 200™ (Luminex Corporation, Austin, Texas, USA).

Los análisis estadísticos se realizaron utilizando el software R. Debido a la distribución anormal de las variables, se utilizó la prueba de U de Mann-Whitney para comparar las variables clínicas cuantitativas y los niveles de IL1beta entre el grupo control y el grupo perio no tratado. La prueba exacta de Fisher se utilizó para evaluar la asociación de las variables cualitativas entre ambos grupos. Tras el tratamiento periodontal, se empleó la prueba de Wilconxon para grupos apareados con la finalidad de contrastar los parámetros periodontales y los niveles de IL1beta entre el grupo perio tratado y el no tratado. El nivel de significación aplicado fue $p<0,05$.

Los modelos predictivos se construyeron seleccionando IL1beta como variable predictiva. Se realizaron dos fases de modelado: en la primera, los pacientes periodontalmente sanos fueron considerados como "condición control"; en la segunda, la "condición control" fueron aquellos pacientes periodontales tratados que mostraron una mejora clínica significativa en términos de BOP y PPD. En ambas fases del modelado, la "condición enfermedad" estuvo representada por los pacientes con periodontitis sin tratar. Los dos modelos se desarrollaron tanto para no fumadores como para fumadores (primer modelado, n=76 y 65, respectivamente; segundo modelado -diseño apareado-, n=60 y 40, respectivamente).

La capacidad de los modelos para discriminar la presencia de periodontitis sin tratar se evaluó utilizando el valor AUC. Cabe señalar que los modelos con un valor de AUC igual o superior a 0,70 se consideran modelos predictivos aceptables [65]. Para validar internamente los modelos predictivos basados en IL1beta, se determinó el valor de optimismo sobre las medidas aparentes de discriminación utilizando métodos "*bootstrap*". El valor bc-AUC se obtuvo a partir de su correspondiente medida aparente derivada de la muestra original menos su respectivo valor medio de optimismo [69,70].

Como medida de calibración de los modelos se aplicó el test de Hosmer-Lemeshow. Los nomogramas fueron construidos gráficamente en base a los resultados del análisis predictivo. El umbral de clasificación óptimo para cada modelo se definió como aquel que

proporcionó el porcentaje máximo de predicciones correctas (máximo ACC). También se calcularon los umbrales diagnósticos para los valores de sensibilidad y especificidad >90% [79].

5.2. RESULTADOS

Los niveles salivales de la IL1beta fueron significativamente superiores en los pacientes del grupo perio no tratada que los del grupo control, tanto en no fumadores como en fumadores (p<0,001). Los niveles de la IL1beta salival en los pacientes periodontales tratados fueron significativamente más bajos que los presentes en estos mismos pacientes antes del tratamiento periodontal, tanto en no fumadores como en fumadores (p<0,001).

En la primera fase de modelado, el modelo predictivo de IL1beta en los no fumadores mostró valores más altos de AUC que en los fumadores (0,830 frente a 0,689). En la segunda fase de modelado, los modelos predictivos presentaron valores de AUC menores que en la primera fase, y fueron ligeramente superiores en los fumadores (0,708 frente a 0,671 en los no fumadores). Por otro lado, la prueba Hosmer-Lemeshow confirmó la calibración adecuada de los modelos.

Con respecto a los nomogramas, los niveles más altos de IL1beta salival se asociaron a una mayor probabilidad de padecer periodontitis sin tratar. Los umbrales diagnósticos asociados al máximo ACC para la IL1beta salival fueron 84,76 pg/ml para no fumadores y 42,78 pg/ml para fumadores. Tras el tratamiento periodontal, los umbrales diagnósticos de IL1beta para distinguir la periodontitis sin tratar de la tratada fueron 163,50 pg/ml y 27,66 pg/ml, respectivamente.

DISCUSIÓN GENERAL

Aunque existe una literatura muy extensa sobre el tópico de los biomarcadores y la periodontitis, realmente no existe ningún análisis sistemático que evidencie con claridad cuales son hasta la actualidad los biomarcadores más investigados en los fluidos orales para el diagnóstico de la periodontitis, y cuáles de ellos muestran una mayor precisión diagnóstica. Motivados por esta ausencia de evidencia, los dos

primeros objetivos de la presente Tesis se basaron en las dos primeras revisiones sistemáticas/metaanálisis de estudios de precisión sobre biomarcadores en el GCF y saliva para el diagnóstico de la periodontitis.

A pesar de ser un tema de gran interés para la comunidad científica, nuestras revisiones ponen de manifiesto que hay una considerable cantidad de literatura de calidad dudosa en este campo y además existe un predominio de observaciones puntuales de una multitud de biomarcadores, tanto en el GCF como salivales.

En el GCF, la molécula más investigada en el campo de la fiabilidad diagnóstica fue la MMP8, seguido de la elastasa, la catepsina y la tripsina. En la saliva, MMP8 también fue la más estudiada, junto con IL1beta, seguidas ambas de IL6, MMP9 y hemoglobina. Sorprendentemente, la segunda revisión sistemática revela una falta de concordancia biológica en el descubrimiento de biomarcadores salivales para el diagnóstico de la periodontitis. Este hallazgo se ve respaldado por el hecho de que los biomarcadores que más se investigan en la saliva no son los más investigados en el GCF (Objetivo 1), excepto MMP8.

Centrándonos en los biomarcadores más estudiados en el GCF (MMP8 y elastasa), considerando una prevalencia del 45% de periodontitis asociada a un amplio espectro de enfermedades [56,57], teóricamente, el test de MMP8 sería más útil o eficaz clínicamente que la prueba de elastasa, mostrando casi un 90% de verdaderos positivos y alrededor de un 83% de verdaderos negativos. Si consideramos que el test de MMP8 en GCF se utilizara como prueba de cribado para decidir a quién se debería remitir para pruebas adicionales [80], habría un 17% de sujetos con periodontitis en los que la prueba no sería capaz de detectar la enfermedad en el cribado inicial y, por otro lado, sólo un 11% de los pacientes se someterían a una exploración periodontal innecesaria.

Centrándonos en los biomarcadores más estudiados en saliva (MMP8 e IL1beta) considerando una prevalencia del 45% de

periodontitis [56,57] y si se utilizaran los tests de IL1beta y MMP8 en saliva como prueba de cribado [80], habría un 67% y un 63%, respectivamente, de sujetos periodontales en los que la prueba podría detectar la enfermedad en el cribado inicial; por otro lado, las pruebas podrían detectar la condición de no periodontitis en un 79% y un 75%, respectivamente, de pacientes no periodontales.

Aunque está aceptado que la inmunopatogénesis de la periodontitis está impulsada por complejas redes dinámicas de interacciones entre citoquinas [81], muy pocos autores han evaluado la presencia simultánea de más de diez citoquinas en el GCF [82-84]. Por otro lado, basándonos en los hallazgos derivados del Objetivo 1, son escasos los trabajos publicados en la literatura sobre el desarrollo de modelos predictivos basados en citoquinas o en ratios de citoquinas en el GCF para el diagnóstico de la periodontitis mediante técnicas apropiadas de modelización predictiva [52]. Los dos siguientes estudios representan las primeras investigaciones en las que se han evaluado y validado internamente modelos predictivos diagnósticos basados en los niveles de 16 citoquinas en el GCF mediante técnicas de modelado predictivo multivariante, diferenciándose por el hábito tabáquico.

Una prueba diagnóstica ideal debería tener valores de precisión predictiva cercanas al 100% [24], y en el Objetivo 3, se obtuvieron seis modelos predictivos ajustados por el hábito tabáquico formados por una o dos citoquinas en el GCF. Estos modelos, estadísticamente validados, obtuvieron una capacidad para discriminar la periodontitis crónica de >0.93. Por lo tanto, se consideraron "modelos predictivos sobresalientes" [65], demostrando que las citoquinas pueden ser biomarcadores excelentes a la hora de distinguir a los pacientes con periodontitis crónica de los individuos con buena salud periodontal.

Sin embargo, existen opiniones contrarias que defienden la idea de que las citoquinas no son lo suficientemente específicas para predecir la periodontitis, y de que sus niveles en el GCF pueden verse afectados por factores locales o sistémicos como el tabaquismo, el consumo de alcohol y el estrés [85,86]. En nuestra opinión, sin embargo, estas afirmaciones son cuestionables frente a la fuerte evidencia sobre la

capacidad predictiva de ciertas citoquinas encontradas en el presente estudio.

La IL1beta fue la que presentó los mejores parámetros predictivos (medidas de clasificación corregidas >92%) en el GCF, seguido de la IL1alfa (medidas de clasificación corregidas >90%) y la IL17A (medidas de clasificación corregidas >87%). En relación a los modelos de dos variables que combinaban IL1alfa, IL1beta e IL17A con citoquinas antiinflamatorias, se encontraron tres modelos ajustados al tabaquismo: IL1alfa + IFNgamma, IL1beta + IL10 e IL17A + IFNgamma (bc-AUC >0,96; medidas de clasificación corregidas >89%). Estos resultados son la primera evidencia de la alta capacidad predictiva de los modelos basados en una citoquina proinflamatoria y otra antiinflamatoria para distinguir a un paciente con periodontitis crónica de otro sano. Por último, este es el primer estudio que proporciona varios nomogramas de aplicación clínica basados en los niveles de citoquinas en el GCF para predecir la probabilidad de tener periodontitis crónica.

El tabaquismo está considerado un factor de riesgo para la periodontitis crónica [86,87], y su influencia en los niveles de algunas citoquinas en el GCF de los pacientes periodontales ha sido señalada por otros autores anteriormente [82,88]. En la presente serie, observamos en los modelos basados en citoquinas que el tabaco aumentaba en un 15-20% la probabilidad de tener periodontitis crónica, lo que nos motivó a efectuar modelos predictivos basados en citoquinas o ratios de citoquinas específicos para pacientes no fumadores y fumadores.

En el cuarto estudio, en relación con las citoquinas individuales, hubo tres modelos que consistían en IL1alpha, IL1beta e IL17A, que presentaron un bc-AUC>0,90 tanto para fumadores como para no fumadores. Según los expertos en la materia [65], estos altos valores de AUC indican que estas citoquinas proinflamatorias tienen una gran capacidad para discriminar la condición de la enfermedad. Los hallazgos sobre la alta capacidad de predicción de la IL1 son consistentes con los descritos anteriormente por Baeza et al. [46],

mientras que los hallazgos de la IL17 representan la primera evidencia de esta citoquina asociada a una fuerte capacidad diagnóstica. Tres modelos basados en ratios (IL1alfa/IL2, IL1beta/IL2 e IL17A/IL2) presentaron un bc-AUC>0,80 tanto para fumadores como para no fumadores. Estos valores de bc-AUC, aunque más bajos que los detectados en las citoquinas individuales, también fueron muy altos, revelando la excelente capacidad de estos ratios de citoquinas para discriminar a los pacientes con periodontitis [65].

Por primera vez en la literatura, hemos definido umbrales específicos de citoquinas en el GCF con potencial diagnóstico de periodontitis para el estatus de no fumador/fumador, detectándose que los fumadores obtuvieron umbrales de diagnóstico más bajos que los no fumadores. Estos hallazgos revelan la conveniencia de diseñar estudios de biomarcadores para predecir las enfermedades periodontales diferenciando por el hábito tabáquico, especialmente si se quieren definir los umbrales diagnósticos. La determinación de estos umbrales específicos podría representar un primer paso en el diseño y construcción de kits diagnósticos de periodontitis para su uso en la práctica clínica.

Partiendo de la premisa que la saliva es el "espejo del cuerpo" y debido a las ventajas inherentes de su recogida, este fluido podría ser útil para la detección y el control de la periodontitis [89]. Razonablemente, un biomarcador que tenga buena capacidad en GCF para la detección de la periodontitis debería mantener este nivel de precisión en la saliva, aunque probablemente disminuya o pueda verse afectado por cambios en su concentración que lo hagan no correlacionar con los niveles gingivales.

La IL1beta fue la primera citoquina cuantificada en el tejido gingival de pacientes con periodontitis crónica [90], y en el objetivo 3, demostramos que la IL1beta es un biomarcador excepcional para distinguir a los pacientes sistémicamente sanos con periodontitis crónica de los individuos periodontalmente sanos. Sin embargo, como se refleja en el Objetivo 2, se han publicado muy pocos estudios sobre la precisión diagnóstica de la IL1beta en saliva, y ninguno de ellos ha

evaluado el impacto del "hábito de fumar" de un paciente sobre esta capacidad diagnóstica [38,48,49,75-78].

Esta hipótesis representó el Objetivo 5, en el que nos centramos en la capacidad diagnóstica de la IL1beta salival, planteando dos fases de modelado (periodontitis no tratada *versus* salud periodontal; periodontitis no tratada *versus* periodontitis tratada con mejoría clínica) y obteniendo modelos específicos para no fumadores y fumadores. Este trabajo representa el primero en la literatura con este enfoque analítico.

En nuestra serie de no fumadores, los niveles salivales de IL1beta mostraron una excelente capacidad para discriminar la periodontitis sin tratar de la salud periodontal; mientras que en pacientes fumadores esta capacidad se redujo notablemente (AUC=0,830 y 0,689, respectivamente). En la segunda fase de modelado (periodontitis tratada con mejoría clínica *versus* periodontitis no tratada), la IL1beta se mantuvo con una capacidad aceptable para discriminar la periodontitis sin tratar de la periodontitis tratada, siendo curiosamente esta capacidad ligeramente inferior en los no fumadores (valores de AUC =0,671 y 0,708 en los fumadores). Probablemente, la capacidad discriminatoria de los pacientes fumadores se encuentra favorecida por el efecto clínico combinado del tabaco y el tratamiento periodontal sobre los niveles salivales de IL1beta.

Por otro lado, los nomogramas derivados de nuestros modelos predictivos mostraron que los niveles salivales de IL1beta que predicen la probabilidad de padecer periodontitis eran mucho más altos en no fumadores que en fumadores. Estos hallazgos confirman nuevamente el efecto inmunosupresor asociado con el tabaco [86] y su impacto en los niveles salivales de IL1beta en diferentes condiciones clínicas. De hecho, coincidiendo con los resultados del Objetivo 4 sobre muestras del GCF [91], el valor del umbral diagnóstico de la IL1beta salival fue mayor en no fumadores que en fumadores en ambas fases del modelado.

CONCLUSIONES

1. Derivado de la revisión sistemática/metaanálisis de los estudios de precisión diagnóstica en el fluido gingival crevicular, la

metaloproteinasa de matriz 8 posee una buena sensibilidad y una excelente especificidad, resultando el biomarcador clínicamente más útil o eficaz para el diagnóstico de la periodontitis de amplio espectro en sujetos sistémicamente sanos. Otras moléculas, como la mieloperoxidasa o varias citoquinas proinflamatorias, se presentan como biomarcadores diagnósticos prometedores, aunque se precisan más investigaciones de alta calidad para confirmar estas observaciones.

2. Derivado de la revisión sistemática/metaanálisis de los estudios de precisión diagnóstica en saliva, las metaloproteinasas de matriz 8 y 9, las interleuquinas 1beta y 6, y la hemoglobina son biomarcadores salivales con buena capacidad para detectar la periodontitis en sujetos sistémicamente sanos; la metaloproteinasa 9 y la interleuquina 1beta también demostraron una buena capacidad para detectar la condición de no periodontitis. La metaloproteinasa 8 y la interleuquina 1beta son los biomarcadores salivales más investigados, y ambos muestran una eficacia clínicamente aceptable para el diagnóstico de la periodontitis de amplio espectro. Otras moléculas, como la cisteína, la proteína inflamatoria del macrófago 1 alfa y el óxido nítrico (y sus metabolitos relacionados), se presentan como biomarcadores salivales prometedores, aunque se precisan más investigaciones de alta calidad para confirmar estos hallazgos.

3. Las interleuquinas 1alfa, 1beta y 17A en el fluido gingival crevicular son biomarcadores extraordinarios para distinguir a los pacientes sistémicamente sanos con periodontitis crónica de los individuos periodontalmente sanos. La capacidad diagnóstica de estas citoquinas proinflamatorias se incrementa con la incorporación de interferón gamma e interleuquina 10. En los nomogramas, los niveles más altos de estas citoquinas proinflamatorias y el ser fumador aumentan la probabilidad de tener periodontitis crónica (función potenciadora), mientras que el interferón gamma y la interleuquina 10 muestran la función opuesta (función protectora). La aplicación clínica de estos biomarcadores podría contribuir a mejorar el diagnóstico y seguimiento de la periodontitis, aunque se necesitan estudios de validación externa para confirmar la aplicabilidad universal de nuestros hallazgos.

4. Las interleuquinas 1alfa, 1beta y 17A, y sus respectivas ratios con la interleuquina 2, son excelentes biomarcadores en el fluido crevicular gingival para distinguir a sujetos sistémicamente sanos con periodontitis crónica de individuos periodontalmente sanos, independientemente del hábito tabáquico. Se definen los umbrales diagnósticos de las citoquinas en fluido, observándose que los fumadores tienen valores umbral más bajos que los no fumadores. Este hecho revela la conveniencia de diseñar estudios de biomarcadores de periodontitis diferenciando por el hábito tabáquico, especialmente si se quieren definir con precisión los umbrales diagnósticos.

5. La interleuquina 1beta en la saliva es un excelente biomarcador para distinguir a los pacientes sanos sistémicamente con periodontitis no tratada de los que están sanos periodontalmente, aunque este potencial discriminatorio se reduce en los fumadores. La capacidad diagnóstica de esta citoquina se mantiene aceptable discriminando entre pacientes con periodontitis no tratada y pacientes con periodontitis tratada con mejoría clínica, especialmente en fumadores. Los umbrales diagnósticos de la interleuquina 1beta salival en fumadores son inferiores a los de los no fumadores en diferentes entornos clínicos, lo que pone de manifiesto la importancia de determinar los umbrales diagnósticos específicos de este biomarcador salival en ambas condiciones de tabaquismo.

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THESIS SUMMARY

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Thesis Summary

"Accuracy of Molecular Biomarkers in Oral Fluids for Diagnosis of Periodontitis"

The purpose of the first two studies (Objectives 1 and 2) was to analyse, using a meta-analytical approach, the diagnostic accuracy of molecular biomarkers in gingival crevicular fluid (GCF) and saliva for the detection of periodontitis in systemically healthy subjects.

Studies on molecular biomarkers in GCF and saliva providing a binary classification table (or sensitivity and specificity values and group sample sizes) in individuals with clinically diagnosed periodontitis were considered eligible. The search was performed using six electronic databases. Meta-analyses were performed using the hierarchical summary receiver operating characteristic modelling, which adjusts classification data using random-effects logistic regression.

In GCF, MMP8 showed good sensitivity and excellent specificity, which resulted in this biomarker being clinically the most useful for the diagnosis of periodontitis in systemically healthy subjects. In saliva, MMP8 and IL1beta were the most researched biomarkers in the field, both showing clinically fair effectiveness for the diagnosis of periodontitis.

The third objective was to obtain GCF cytokine-based predictive models and develop nomograms derived from them.

A sample of 150 systemically healthy participants was recruited, including 75 periodontally healthy controls and 75 subjects affected by

chronic periodontitis. Sixteen mediators were measured in GCF using the Luminex 100™ instrument: granulocyte-macrophage colony-stimulating factor -GMCSF-, interferon -IFN- gamma, interleukin -IL-1alpha, IL1beta, IL2, IL3, IL4, IL5, IL6, IL10, IL12p40, IL12p70, IL13, IL17A, IL17F and tumour necrosis factor -TNF- alpha. Cytokine-based models were obtained using multivariate binary logistic regression and adjusted by smoking status.

IL1alpha, IL1beta and IL17A in GCF are outstanding biomarkers for distinguishing systemically healthy patients with chronic periodontitis from periodontally healthy individuals. The predictive ability of these pro-inflammatory cytokines was increased by incorporating IFNgamma and IL10. The nomograms revealed the amount of periodontitis-associated imbalances between these cytokines with pro-inflammatory and anti-inflammatory effects in terms of a particular probability of having chronic periodontitis.

The fourth objective was to determine cytokine thresholds derived from predictive models for the diagnosis of chronic periodontitis, differentiating by smoking status.

The patient series and cytokine determinations correspond to those described in Objective 3. The models were obtained using binary logistic regression, distinguishing between non-smokers and smokers. The AUC and numerous classification measures were obtained. Model curves were constructed graphically, and the cytokine thresholds calculated for the values of maximum accuracy.

IL1alpha, IL1beta and IL17A, and their ratios with IL2 appeared to be excellent diagnostic biomarkers in GCF for distinguishing systemically healthy subjects with chronic periodontitis from periodontally healthy individuals. Cytokine thresholds in GCF with diagnostic potential are defined, showing that smokers have lower threshold values than non-smokers.

The fifth objective was to obtain salivary IL 1beta-based models to predict the probability of the occurrence of periodontitis, differentiating by smoking habits.

A total of 141 participants were recruited, including 62 periodontally healthy controls, as well as 79 subjects affected by periodontitis. Fifty of those in this latter group were given non-surgical periodontal treatment and showed significant clinical improvement at two months. IL1beta was measured in the salivary samples using the Luminex 200™ instrument. Predictive models were obtained using binary logistic regression to differentiate untreated periodontitis from periodontal health (first modelling) and untreated periodontitis from treated periodontitis (second modelling), distinguishing between non-smokers and smokers. The AUC and numerous classification measures were obtained.

Salivary IL1beta has an excellent diagnostic capability when it comes to distinguishing systemically healthy patients with untreated periodontitis from those who are periodontally healthy, although this discriminatory potential is reduced in smokers. The diagnostic capability of salivary IL1beta remains acceptable for differentiating between untreated and treated periodontitis. The diagnostic threshold values of salivary IL1beta in smokers are lower than in non-smokers in different clinical settings.

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INTRODUCTION

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Introduction

I.1. Concept of Periodontal Diseases

Periodontal diseases are pathologies concerning tissues surrounding the teeth. They could be defined as chronic and multifactorial diseases initiated by an imbalance between the subgingival microbiota and the host homeostasis [1]. Periodontal diseases, along with dental caries, represent the two most prevalent diseases of the mouth [2] and are among the most prevalent diseases worldwide [3].

Periodontitis, the most severe condition of periodontal diseases, is characterised by the destruction of the tooth-supporting structures [4]. Periodontitis usually starts from its mildest and reversible form, gingivitis [5]. Gingivitis originates when bacterial plaque deposited on tooth surfaces causes an inflammatory response in oral soft tissues. Clinically, gingivitis is characterised by swelling of the gums and usually bleeding, especially during brushing or flossing. However, once optimal oral hygiene is carried out, the disruption of gingival tissues is repaired by the disappearance of the oral biofilm. Thus, gingivitis is considered a reversible condition [6].

On the other hand, if the inflammatory condition is maintained, in susceptible individuals, the accumulation and maturation of bacterial plaque may lead to periodontitis, which is characterised by periodontal tissue breakdown. The ulceration of the gingival sulcus becomes deeper due to the disruption of connective tissue fibres (periodontal ligament); bone destruction causes one of the main signs of periodontitis: periodontal pockets (Figure 1). Periodontitis and gingivitis have typical signs and symptoms such as gum inflammation with redness and bleeding, excess tartar, halitosis and increased tooth sensitivity.

Periodontal pockets, dental mobility and eventually, tooth loss are particular signs of periodontitis. Like gingivitis, periodontitis is mainly painless, so these diseases can advance to severe forms before they are detected [5].

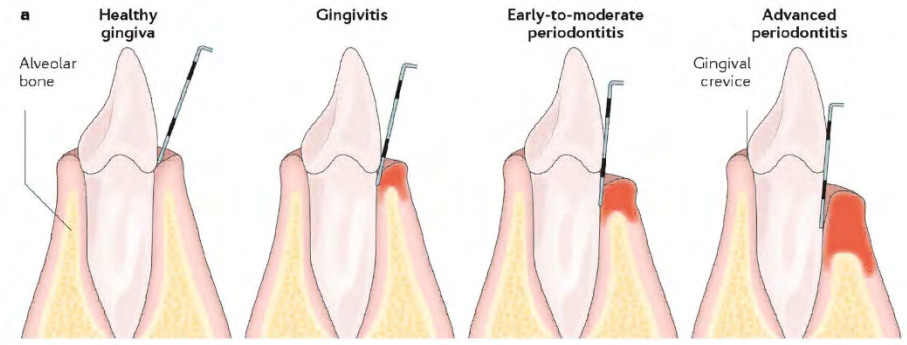

Figure 1. The main stages of periodontal diseases. The image was taken from Kinane et al. [5] with permission of Springer Nature.

I.2. EPIDEMIOLOGY OF PERIODONTAL DISEASES

Periodontitis is recognised as one of the most prevalent chronic diseases in humans worldwide [7]. Furthermore, in 2010, severe periodontitis was estimated as the 6th most prevalent disease in the world [8]. Periodontitis affects over 50% of the adult population and reaches 11% in its severe forms [7,9]. In the USA, the prevalence of periodontitis was 47% in adults older than 30 years old, which means about 65 million people [10]. In Europe, above the age of 60 years old, this percentage increases to 70-85% [11].

Periodontitis is a plaque-induced multifactorial disease with multiple risk factors: local, systemic, congenital and socio-economic factors (Figure 2) [12]. Congenital factors are those that cannot be modified, such as sex or genetics. For instance, according to the literature, periodontitis appears to be significantly more prevalent in men than in women [10,13], although variation in dietary protein content could influence this difference [14]. Furthermore, it has been suggested that the heritability of periodontitis is around 50%. Several

polymorphisms have been identified that appear to be essential for periodontitis predisposition in its beginning and progression [5,15].

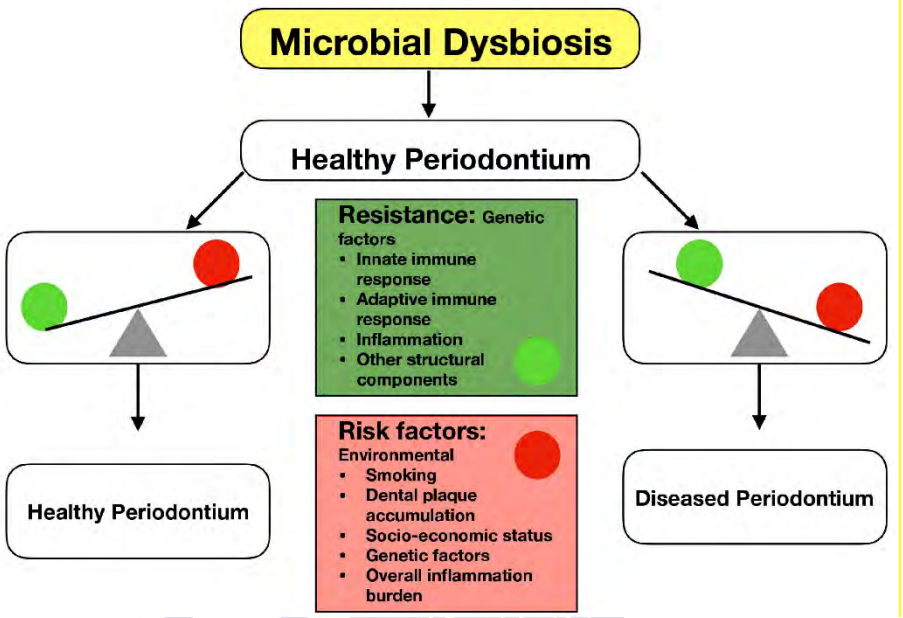

Figure 2. Susceptibility to periodontal diseases. The image was adapted from Kinane et al. [5] with permission of Springer Nature.

The genomic associations between polymorphisms and periodontitis are still being investigated since there is no substantial evidence so far. Possibly, there is not a single gene involved but many that are interacting with each other. This fact, along with some other environmental and epigenetic factors, ends up developing periodontitis [5].

As we mentioned, periodontitis is a dental-plaque-bacteria induced disease, so poor oral hygiene is a crucial local factor [16]. Poor oral hygiene is, in turn, directly related to age, since as it increases, psychomotor skills are gradually lost, and it becomes more difficult to brush or to use floss properly [3].

However, genetic factors play a key role, since periodontitis, as opposed to gingivitis, does not occur in all people who have poor hygiene [17]. This resilience of some individuals to develop periodontitis may be caused not only by genetic mechanisms but also by microbiological ones. Among these microbiological mechanisms, it is worth highlighting the resistance to plaque accumulation due to antimicrobial compounds or salivary nitrate concentrations; and suppression of inflammation due to microbiota (e.g. some species such as *Neisseria* and *Streptococcus* are correlated with anti-inflammatory markers) [18].

Nevertheless, there are other risk factors susceptible to change. Among these are included: bad habits of oral hygiene, as we mentioned before, tobacco [19], alcohol [20] and stress [21]. All of them are modifiable factors and patient-dependent, which complicates the correct handling of the disease [5].

Tobacco and its relationship with periodontal diseases have been widely studied in the literature. Cigarette smoking has been associated not only with tooth loss but also with a dose-response increase risk of suffering periodontitis [19,22]. A meta-analysis of 2,361 subjects showed that smoking habit was associated with periodontitis with an odds ratio (OR) of 2.82 [23]. Furthermore, studies have demonstrated that smokers have higher progression rates of periodontitis than non-smokers. Smoking affects periodontal healing, the gain in attachment loss or the reduction in periodontal pockets are less in smokers than in non-smokers [24]. The most significant scientific evidence comes, however, from those studies that suggest that one of the most important measures in the management of periodontitis is the smoking cessation [25]. Tobacco has also been linked to the inhibition of various inflammatory markers, which is consistent with the low levels of clinical inflammation in smokers [26]. Besides, tobacco might induce changes in oral microbiota, causing different microbiological profiles in smokers. It has been suggested that tobacco may increase the susceptibility to colonisation pathogens responsible for biofilm formation [27-29].

Although alcohol has not been studied as much as tobacco, it has been hypothesised that the risk of periodontitis is also dose-dependent [20]. Additionally, although not so studied, correlations between periodontitis and clinical stress syndrome, work tension or economic problems have been established, suggesting that stress can also be a risk factor for periodontitis [21].

Socio-economic status of patients has also been shown to influence oral health status through various studies. To give an example, Manski and coworkers [30] associated income and education level with visits to the dentist, while Kocher et al. [31] pointed out that a higher educational level acted as a protector against periodontitis and tooth extraction. Another paper revealed that people from lower socio-economic status used home remedies avoiding professional care, while upper socio-economic subjects complemented self-care with professional care [32].

Finally, as we will see later, different systemic conditions such as endocrine or other inflammatory diseases also influence the appearance of periodontitis [33].

I.3. Quality of Life and Periodontitis

According to literature, periodontitis is not a "silent" problem. Regarding the quality of life, it has been demonstrated that periodontal patients have a more reduced perception of their oral health and worse quality of life as compared to healthy individuals [34]. Quality of life is defined as "the degree to which a person enjoys the important possibilities of life" [35]. Advanced periodontitis could compromise different daily life functions, including mastication or even speaking due to pronunciation difficulties [36-38]. Besides, since it usually leads to tooth loss, periodontitis is associated with worse nutrition, caused by the reduction of food diversity [39,40]. In turn, this effect has been correlated with hospital visits and morbidity in the elderly [41].

Last but not least, it is the fact that the smile's aesthetics could be affected in advanced periodontitis, due to tooth loss or gum recessions, which would lead to a decrease in self-esteem and even to worse

employment expectations [32,42]. For years, people have been living longer and considering that they also expect a higher quality of life [10]. Because of this, the prevention and treatment of periodontitis are increasingly important aspects from this point of view.

Shanbhag et al. [43] carried out a systematic review concluding that conventional periodontal treatment (scaling and root planing, SRP) can improve the oral health-related quality of life of periodontitis patients. In 2016, Santuchi et al. [44] conducted a clinical randomised controlled trial where the impact on oral health-related quality of life of two types of periodontal treatments (SRP and one-stage full-mouth disinfection) were analysed. Although there were no differences between them, both groups improved their oral health-related quality of life.

I.4. DIAGNOSIS OF PERIODONTAL DISEASES

Despite all the information published, there is a lack of consensus on a specific definition of periodontitis in the literature [45]. Most of the authors based the diagnosis only on clinical criteria, but the measurements used vary between studies. Colour of gums, gingival recessions or periodontal pocket depth are some of the signs commonly tested to assess periodontal diseases. Other authors also include radiographic evidence of bone loss in the diagnostic criteria. It has also been affirmed that one way of monitoring disease activity would be by measuring bleeding on probing (BOP), considering healthy controls that ones where the bleeding appear in less than 20-25% of the gingival sites [46].

I.4.1. Classifications of Periodontal Diseases

Over the years, multiple authors have been developing different classifications of periodontal diseases, but in this Thesis, we will focus on the most important, to the best of our knowledge.

I.4.1.1. Classification of Armitage

In 1999, for the Classification Workshop of the American Academy of Periodontology, Dr Armitage developed a comprehensive classification, much of which is still used today. Periodontal diseases were classified into two categories: gingivitis (gum inflammation without attachment loss) and periodontitis (gum inflammation with attachment loss/bone destruction) (Table 1) [47].

Table 1. Overview of the classification of periodontal diseases by Armitage (1999) [47].

Classification of Periodontal Diseases and Conditions	
I. Gingival diseases	A. Dental plaque-induced gingival diseases
	B. Non-plaque-induced gingival lesions (drugs)
II. Chronic periodontitis	A. Localised
	B. Generalised (>30% of sites are involved)
III. Aggressive periodontitis	A. Localised
	B. Generalised (>30% of sites are involved)
IV. Periodontitis as a manifestation of systemic diseases	A. Associated with haematological disorders
	B. Associated with genetic disorders
	C. Not otherwise specified
V. Necrotising periodontal diseases	A. Necrotising ulcerative gingivitis
	B. Necrotising ulcerative periodontitis

VI. Abscesses of the periodontium	A. Gingival abscess
	B. Periodontal abscess
	C. Pericoronal abscess
VII. Periodontitis associated with endodontic lesions	A. Combined periodontic-endodontic lesions
VIII. Development or acquired deformities and conditions	A. Localised tooth-related factors that modify or predispose to plaque-induced gingival diseases/periodontitis
	B. Mucogingival deformities and conditions around teeth
	C. Mucogingival deformities and conditions on edentulous ridges
	D. Occlusal trauma

The table was modified from Armitage (1999) [47].

The classification also divided periodontitis in chronic, that will be the focus of this Thesis, and aggressive. Aggressive periodontitis combined the terms "juvenile periodontitis" and "early-onset periodontitis" that had been used to date. Aggressive periodontitis is characterised by a rapid progression of the disease; contrary to chronic periodontitis that usually occurs in middle-aged people, affects people under 30 years old [48]. Furthermore, both gingivitis and periodontitis could be initiated not only by bacterial factors but also for other factors such as systemic diseases or endodontic lesions (Table 1).

Periodontitis could also be divided according to the extent (number of teeth affected) as localised and generalised. According to the author [47], periodontitis could be characterised as "localised" if up to 30% of the teeth were involved and as "generalised" if >30% of the teeth are affected.

I.4.1.2. Classification of Page and Eke

In 2007, Page and Eke proposed a disease category by the severity of the periodontitis (amount of loss of periodontal tissue) measured as clinical attachment loss (CAL) and probing pocket depth (PPD) (Table 2). Both parameters are measured with a periodontal probe.

Table 2. Overview of the classification of periodontitis proposed by Page and Eke (2007) [49].

Disease Category	Clinical Definition		
	CAL		PPD
Severe Periodontitis	≥2 interproximal sites with CAL ≥6 mm (not in the same tooth)	and	≥1 interproximal site with PPD ≥5 mm
Moderate Periodontitis	≥2 interproximal sites with CAL ≥4 mm (not in the same tooth)	or	≥2 interproximal site with PPD ≥5 mm (not in the same tooth
No or Mild Periodontitis	Neither "moderate" nor "severe" periodontitis		

*Third molars are excluded.

PPD is the distance from the gingival margin to the base of the gingival sulcus (or the periodontal pocket in case of periodontitis) while CAL is the distance from the cemento-enamel junction to the base of the gingival sulcus. Page and Eke [49] proposed that although CAL is more reliable to measure the progression and severity of the disease, using it alone could lead to confusion because the attachment loss may be due to non-inflammatory factors (i.e. healthy sites with recessions). On the other hand, using PPD alone could also take to false results: in young people, CAL is equal to PPD, but after middle age, PPD decreases because recessions increase.

Flemmig [50] classified periodontitis similarly. This author called "generalised periodontitis" when the disease affects >10 of the 32 teeth and "localised periodontitis" when it affects fewer teeth.

I.4.1.3. New Classification of Periodontal Diseases

After 19 years, a consensus report from experts all over the world gave rise, in 2018, to an updated classification of periodontal diseases (Table 3).

Table 3. New classification of periodontal diseases (2018) [51,52].

New Classification of Periodontal Diseases and Conditions	
I. Necrotising periodontal diseases	A. Necrotising gingivitis
II. Periodontitis as a manifestation of systemic disease	Classification of these conditions should be based on the primary systemic disease according to the International Statistical Classification of Diseases and Related Health Problems (ICD) codes
III. Periodontitis	A. Stages based on severity (CAL at the site with the most significant loss, radiographic bone loss and tooth loss) and complexity of management (PPD, patterns of bone loss, furcation lesions, number of remaining teeth, tooth mobility): -Stage I: initial periodontitis -Stage II: moderate periodontitis -Stage III: severe periodontitis with potential for additional tooth loss -Stage IV: severe periodontitis with potential for loss of the dentition
	B. Extent and distribution: localised (<30% of sites are involved); generalised (>30% of sites are involved); molar-incisor distribution
	C. Grades: evidence or risk of rapid progression, anticipated treatment response: -Grade A: slow rate of progression -Grade B: moderate rate of progression -Grade C: rapid rate of progression

In this new classification, the authors differentiated between three forms of periodontitis: “necrotising periodontitis”, “periodontitis as a manifestation of systemic disease" and “periodontitis” (this category comprising the classic concepts “chronic” and “aggressive”) [51,52]. Due to the nature of this Thesis, we will only focus on the last one, although the three conditions are represented in Table 3. The classification is characterised on the one hand, in different stages based on the severity and the complexity of disease management; and on the other hand, in grades based on the risk of rapid progression and the individual patient factors, such as smoking habits or hyperglycemia, which are used as grade modifiers [51,52].

I.4.2. Periodontal Diseases and Oral Indices

Epidemiological indices have been developed to quantify the clinical stages of oral health and to facilitate the comparison between different populations. These indices should be able to collect information objectively, be reproducible and easy to use.

There are numerous types of oral indices, some of which are responsible for evaluating plaque or dental calculus, while others analyse gingival inflammation or caries [53]. One of the first indices was developed by Ramfjord 60 years ago [54]. Ramfjord designed a numerical scale to evaluate different aspects of periodontal health, such as the presence and extension of plaque or calculus covering tooth surfaces or the depth of the gingival sulcus/periodontal pocket. Although the first two components are not part of the Periodontal Disease Index, they are considered to provide relevant information for the total evaluation of the periodontal condition.

In order not to overextend this introduction, we will present below a table-summary of the main periodontal indices described in the literature (Table 4).

Table 4. Overview of periodontal indices [51,52].

Indices	Selected Teeth	Criteria	Guidelines
Periodontal Disease Index [54]	Six teeth: 16, 21, 24, 36, 41 and 44	0-3: if the gingival sulcus does not extend to the apex beyond the amelo-cementary limit, the 0-3 score recorded for gingival health status is considered to be the periodontal disease index of the tooth 4: if the depth of the pocket extends to the apex beyond the amelo-cementary limit, but not more than 3 mm in any of the four areas examined 5: if the pocket extends to the apex more than 3 mm and up to 6 mm from the amelo-cementary limit 6: if the distance between the amelo-cementary limit and the bottom of the pocket is greater than 6 mm along the root	Has been used successfully in longitudinal epidemiological studies of periodontitis and clinical trials of preventive and therapeutic agents
Extension and Severity Index (ESI) [55]	28 locations: centre of the buccal surface and mesiobuccal angle of the seven teeth of the right upper quadrant and the seven of the lower left quadrant	The result is expressed in two numbers: the first indicates the percentage of areas with signs of disease and the second the average depth of the pockets	Simple and reproducible Underestimation of the severity
Mobility Index [54]	Six teeth: 16, 21, 24, 36, 41 y 44	M0: physiologic mobility; firm tooth M1: slightly increased mobility M2: definite to considerable increase in mobility, but no impairment of function M3: extreme mobility; a "loose" tooth that cannot be used for normal function	In disuse
CPITN-Community Periodontal Index of Treatment Needs [56]	All teeth of a sextant or only index teeth (17, 16, 11, 26, 27, 36, 37, 31, 46 and 47)	0: healthy 1: sextant without pockets, tartar or overflowing restorations, but in which there are bleeding after a soft catheter in one or more locations 2: sextant in which there are no pockets that exceed 3 mm, but in which the tartar and plaque are	Designed to discriminate treatment needs in large population groups, and to facilitate preventive and

Indices	Selected Teeth	Criteria	Guidelines
In 1997, it was modified adding the parameter CAL		visible or are recognised subgingivally 3: sextant with pockets of 4 or 5 mm 4: sextant with pockets of ≥6 mm X: sextant excluded (less than 2 teeth present) 9: not registered	therapeutic strategies
Periodontitis Index [57]	Number (or percentages) of teeth showing thresholds of PPD	Classifies as no-periodontitis, mild, moderate or advanced periodontitis, and attachment loss	
Genetic Susceptibility Index for periodontal diseases [58]		Both single nucleotide polymorphisms (SNPs) and microbial components of periodontal diseases	Further studies are required to validate and apply it

I.5. Treatment of Periodontal Diseases

Treatment of periodontal diseases is based on the elimination of oral biofilm to restore host homeostasis. It is necessary to establish a biofilm control through the implementation of correct oral hygiene. There are two types to control oral biofilm: mechanically (tooth brushing and flossing or professional prophylaxis) and chemically (oral antiseptics and antibiotics).

I.5.1. Mechanical Treatment

Conventional treatment of periodontitis, SRP, consists of mechanical removal of the bacterial plaque, tartar and contaminated cementum attached to the root surface. This removal is done using manual (curettes) or ultrasonic instruments and sometimes aided by the irrigation of antiseptics (chemical control). The purpose of SRP is to eliminate the microbiota disrupting homeostasis to reestablish the microbiological and chemical balance. Logically, deep pockets are more difficult to scale [59], so in pockets greater than 6 mm and furcations, SRP may not be enough to control the disease [60]. In these cases, an open flag debridement is usually performed. Open flag periodontal surgery consists of surgically separating a specific section of the gingiva in order to have visibility to deep pockets. The success

of the chosen treatment depends both on the operator's ability and motivation and maintenance of patients in their oral hygiene [5].

I.5.2. Chemical Treatment

Over the years, several treatment strategies have been studied to manage periodontal diseases. The use of oral antiseptics, as well as administration of systemic antibiotics, are considered essential adjuvants to non-surgical periodontal treatment.

In the chemical control against oral biofilm, the gold standard is chlorhexidine. It was the first antiseptic capable of inhibiting plaque formation and its effectiveness towards periodontal pathogens has been extensively researched. The main advantages of chlorhexidine include low toxicity, the capacity of being bacteriostatic or bactericidal (at low or high concentrations, respectively) and its substantivity, which is the property of adhering to a wide variety of substrates maintaining its antimicrobial activity [61-64].

Other well-known antiseptics that have demonstrated its antibacterial effectiveness are essential oils. They were used since ancient times, but it was not until the late 1870s when they were popularised [65]. Curiously, in an *in situ* study published in 2015, where the antimicrobial activity between a single application of an essential oil mouthwash *versus* a chlorhexidine mouthwash was compared, the results were better for essential oils. Furthermore, the essential oil mouthwash maintained its antibacterial activity at least for seven hours [66].

As we mentioned, the achievements of periodontal treatment are to eliminate inflammation and reduce bacterial burden. For some time, it has been suggested that the combination of systemic antibiotics with non-surgical periodontal treatment could suppress pathogenic bacteria to return to a healthy clinical condition [67].

Herrera et al. [68] and Haffajee et al. [69] conducted two systematic reviews to evaluate the effectiveness of antibiotherapy adjunctive to SRP. These authors concluded that, although there were

methodological discrepancies between studies, systemic antimicrobials may offer additional advantages over SRP alone, improving the outcomes in deep pockets. Herrera et al. [68] included 25 controlled clinical trials with a follow-up of six months, where periodontal systemically healthy patients were treated with or without systemic antibiotics. The outcomes were better in those who were treated with antibiotics. Haffajee et al. [69] included 27 controlled clinical trials with a follow-up of at least one month, but the conclusions were quite similar to those of Herrera and coworkers [68]. More recent evidence has also indicated that some antibiotics, amoxicillin in combination with metronidazole or simply metronidazole, can improve clinical outcomes of periodontal treatment comparing with SRP alone [67]. It has been recommended that the combination of amoxicillin and metronidazole is appropriate for around 70% of periodontitis patients. Commercially available methods, such as ParoCheck® chip, allow the rapid detection of many periodontopathogens, allowing a more personalised treatment [70].

Furthermore, there is increasing evidence that non-surgical periodontal treatment can also have an impact on systemic health. For example, multiple studies have shown that glycated haemoglobin levels (HbA1c) in diabetes type II patients decrease after periodontal treatment [71,72]. A later section will explain this topic in greater detail.

Finally, we would like to mention other adjuvant therapies in periodontal treatment such as the laser (diode and photodynamic therapy) and local delivery of drugs (chlorhexidine chips, doxycycline and minocycline gels and doxycycline microspheres). All of them have proven their clinical benefits compared with SRP alone in different systemic reviews, specifically in deep pockets or recurring disease; although the effects of laser-assisted SRP over pro-inflammatory cytokines remains yet unclear [73-75].

I.6. RELATIONSHIP BETWEEN PERIODONTITIS AND SYSTEMIC DISEASES

The oral cavity is not an isolated part of the human body but a part of the biological processes taking place in it [3,76]**.** Since years ago, it has been stated that periodontitis is related to different systemic diseases in a two-way relationship. This link has led to the development of a new branch of Dentistry called Periodontal Medicine [77]. Periodontal Medicine appeared when scientific evidence showed a bidirectional relationship between periodontitis and some systemic diseases such as cardiovascular disease [78], diabetes [79] or rheumatoid arthritis [80]. In recent years there have also found numerous epidemiological associations with respiratory diseases [81], adverse pregnancy outcomes [82] or even Alzheimer's disease [83].

Different mechanisms of action have been proposed as hypotheses by which periodontitis may affect the rest of the body. In general, we could differentiate three main pathogenic pathways (Figure 3):

1. Metastatic infection, which consists of the direct effect of oral microorganisms or part of them (for instance, antigenic cell walls) to other tissues and organs. This dissemination can occur in three different ways a) by the direct spreading of oral bacteria to other parts of the body through contiguous spaces or venous or lymphatic drainage (e.g. brain abscess); b) by aspiration, what would explain the respiratory pathologies; and c) by bacteraemia or access of the oral bacteria to the bloodstream. In this last one, endocarditis is the most representative infection.

2. Metastatic lesion, which consists of the indirect effect of bacterial toxins on tissues or organs, developing, for instance, endotoxemia.

3. Metastatic inflammation, which consists of the host systemic immune response against oral microorganisms and their virulence factors [84].

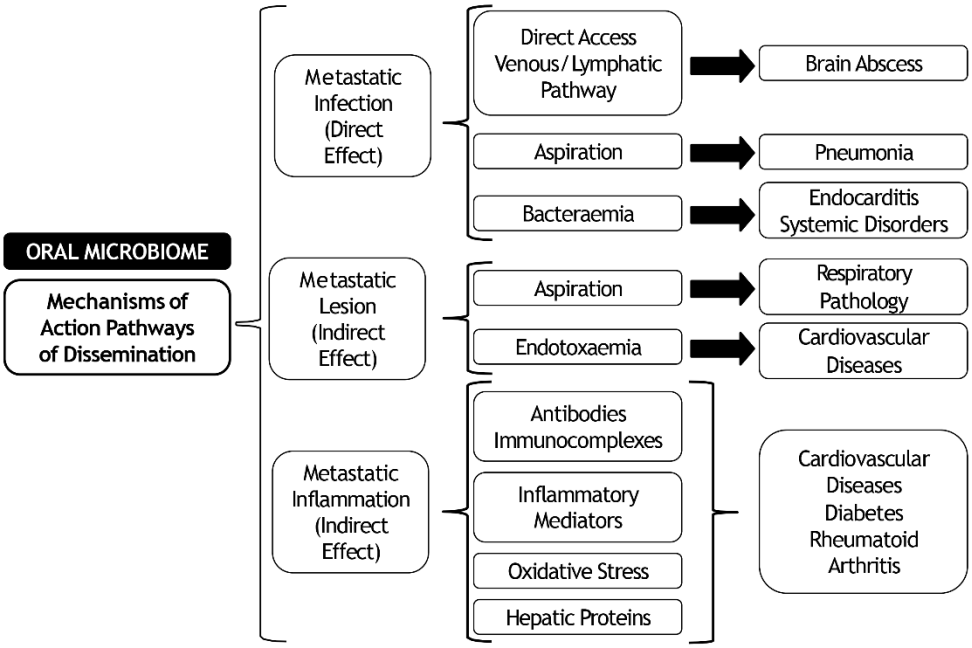

Figure 3. Mechanisms of action/pathways of dissemination.

We will proceed to give a summary of the relationship between periodontitis and some systemic diseases. We are aware that there are many more diseases that could be linked to periodontitis, such as obesity, cancer or chronic kidney disease, but in this Thesis, we want to focus on those that have been more studied.

I.6.1. Periodontitis and Cardiovascular Diseases

Since 1980, numerous studies have been carried out to investigate this possible association [85]. Cardiovascular diseases (CVDs), which include, among others, ischaemic heart disease, peripheral vascular disease or cerebrovascular diseases, are the leading cause of death worldwide [86].

Scientific evidence shows a clear but weak correlation between periodontitis and CVDs. Periodontitis was reported to increase the risk of future CVDs events independently of other factors [87], but also, it

seems that people suffering from CVDs have worse periodontal health than those who do not [88]. Bahekar et al. [89] conducted a meta-analysis of five prospective cohort studies (86,092 patients) and found a 1.14 higher risk of suffering coronary heart disease than controls. In the meta-analysis of the case-control studies (1,423 patients) the risk increased to 2.2, and in the five cross-sectional's (17,724 patients), the risk of coronary heart disease was 1.52 compared to those without periodontal pockets. In another meta-analysis, the risk ratio was 1.24 in patients with periodontitis [90]. Humphrey's meta-analysis [90] concluded that periodontitis is an independent but weak risk factor for CVDs. In a more recent systematic review of 12 studies (cohort and case-control), carried out by Dietrich et al. [91], only one of the studies [92] found no positive associations between PPD and CVDs. Dietrich and coworkers [91] concluded that the association between periodontitis and CVDs was stronger in younger adults compared to older subjects (over 65 years). Another study with 8,446 patients and 13 years of follow-up revealed that few missing teeth might indicate an increased risk of CVDs [93].

The mechanisms that link both diseases remain unclear. In normal conditions, the epithelial barrier of the periodontium and the protective immune molecules prevent periodontopathogens from entering into the bloodstream. It has been suggested that periodontal bacteria or their products (e.g. lipopolysaccharides, LPS) could pass into the bloodstream through the ulcerated tissue of the periodontal pockets. Activation of the host inflammatory response is then produced by several mechanisms, which contributes to the formation and maturation of atheroma (Figure 3) [94,95]. Among the periodontal pathogens that have been related to atherosclerosis are *Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Tannerella forsythia, Fusobacterium nucleatum* and specially *Porphyromonas gingivalis,* that has been well studied due to its capacity of invading the endothelial cells [95].

In a systematic review of 2008, the results showed that patients with periodontitis had elevated C-reactive protein (CRP) serum levels than healthy subjects [96]. Other studies have confirmed that CRP is

also elevated in patients with aggressive periodontitis [97,98]. CRP is an inflammatory marker closely related to heart disease and a value greater than 3 mg/ml of CRP is considered high risk for CVDs [99].

Conventional periodontal treatment may contribute to improving the outcome of CVDs by decreasing circulating CRP levels in a range from 0.2 mg/ml to 0.5 mg/ml [72,96]. However, further studies are needed because another systematic review concluded that there were no papers which examined the impact of non-surgical periodontal treatment in the primary prevention of CVDs in periodontitis patients [100].

I.6.2. Periodontitis and Diabetes Mellitus

Diabetes mellitus is a metabolic disorder, characterised by the constant presence of high blood glucose levels, due to defects in insulin production, insulin action or a combination of both [101]. Diabetes affects an estimated 346 million people worldwide and is becoming a significant public health problem and a global epidemic [102].

The relationship between diabetes and periodontitis has been studied since the 1960s [103]. Nowadays, there is sufficient evidence to support that diabetes is a risk factor for periodontitis, since high blood glucose levels contribute to worse periodontal status [79]. Data have been indicating that higher levels of HbA1c, a marker used to measure glycaemia and diabetes outcome, are associated with a more significant attachment loss [104,105]. But not only diabetes contributes to the severity and progression of the disease, but periodontitis is also a risk factor for the development of diabetes and its future complications [106]. For instance, in a case-control study with a six-year follow-up, periodontal patients had more prevalence of cardiovascular complications and proteinuria than controls [105].

The pathogenic mechanisms linking diabetes and periodontitis are mainly based on inflammation. The inflammatory processes in diabetic people are up-regulated; with the same periodontal status, interleukin (IL) 1beta and tumour necrosis factor (TNF) alpha are more elevated in

gingival crevicular fluid (GCF) in diabetic patients than in non-diabetic patients [107].

Finally, it should be noted that, as discussed earlier, evidence suggests that chronic inflammation from periodontitis may impact diabetes type 2 altering HbA1C levels. On the other hand, mechanical periodontal therapy can also decrease HbA1c levels and therefore improve diabetes outcomes due to HbA1c itself, which is a measure of diabetes control outcomes. Engebretson and Kocher, in a systematic review, observed that a mean reduction in HbA1c of 0.36% (95% confidence interval -CI- 0.19, 0.54) at three months was produced after periodontal treatment [108]. Another systematic review from 2014 also showed a reduction in HbA1c levels after three months of periodontal therapy, although this effect could not be observed beyond three months [109].

I.6.3. Periodontitis and Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune systemic inflammatory disease characterised by persistent and progressive inflammation of the joints that usually ends with structural damage and loss of function [80].

In many cases, patients suffering from RA can result in activity limitations and disability, leading to poor oral hygiene that, in turn, favours periodontitis [33]. Several studies all over the world demonstrate that patients with RA have a higher prevalence and have more severe periodontitis than non-RA people [110,111]. Data from the USA National Health and Nutrition Examination Survey (1988-1994) revealed that the risk of periodontitis was 1.8 (95% CI) in RA patients [110]. On the other hand, periodontal pathogens such as *P. gingivalis* and *P. intermedia* have been found in the serum and synovial fluid of patients with RA, and TNFalpha has also been correlated with the disease [112,113]. Furthermore, the levels of antibodies against *P. gingivalis* have been correlated with the level of anti-citrullinated protein antibodies (ACPA), a specific antibody for RA, in patients with RA [80]. On the other hand, evidence supports that a reduction of some

inflammatory biomarkers due to periodontal treatment may decrease the severity of RA [112,114].

Nevertheless, a prospective study with a follow-up of 12 years found no significant association with the incidence of RA, so more studies are necessary to be able to assure that periodontitis is a risk factor for RA [115].

I.6.4. Periodontitis and Respiratory Diseases

In 2004, chronic obstructive pulmonary disease was the fourth leading cause of death in the USA [116]. Pneumonia is the second most common type of nosocomial infection and accounts for 15% of all infections acquired in the hospital setting [117]. Aspiration pneumonia is an infection of the lungs caused by aspiration of oral bacteria [72].

In general, most authors agree that periodontopathogenic burden is a risk factor for the development of pneumonia since several periodontal microorganisms, such as *P. gingivalis* and *A. actinomycetemcomitans*, have been associated with it [118,119]. Apart from the aspiration of periodontal pathogens, other possible pathways in which oral bacterial agents may participate in the pathogenesis of respiratory diseases are being considered (Figure 4):

1. The colonisation of the respiratory tract by oral pathogens due to the presence of salivary enzymes.

2. Neutrophils and enzymes associated with periodontitis may modify mucosal surfaces to promote adhesion and colonisation by respiratory pathogens (hydrolytic enzymatic effect).

3. Pro-inflammatory cytokines from periodontium may alter the respiratory epithelium to enter the systemic circulation by diffusion [81,120].

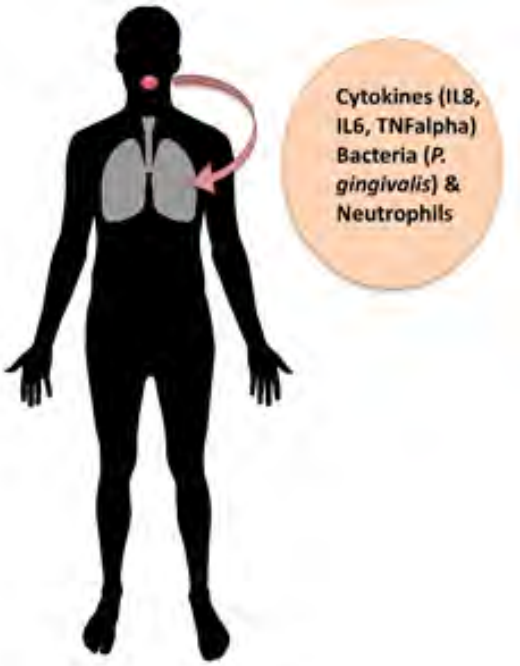

Figure 4. Possible oral modifiers in the pathogenesis of respiratory diseases.

In a 2009 study conducted in a hospital setting, the authors [121] identified that periodontal patients have a 3-fold increased risk of having lower respiratory tract infections than those with a healthy periodontium. The relationship between oral health and chronic obstructive pulmonary disease (COPD) has also been the subject of numerous studies. Some authors argue that this relationship is inconsistent [122,123] and that many COPD patients are smokers.

However, other studies have revealed that severe CAL associated with chronic periodontitis is frequent in patients with COPD. On the other hand, respiratory compromise is aggravated by inadequate oral hygiene and an increase in the number of sites with dental plaque and gingival bleeding [124,125].

The systematic review of Scannapieco et al. [126] indicated that there is an association between periodontal disease and pneumonia and a possible association between periodontitis and COPD. Also, these authors noticed that the incidence of nosocomial pneumonia was reduced by 40% when chemical or mechanical disinfection measures were applied to the oral cavity, as well as the administration of antibiotics. Although Azarpazhooh and Leake [122], in the meta-analysis conducted in 2006, identified nine clinical trials in which the incidence of pneumonia was reduced after an improvement in oral health indicators; in another study of 30 patients periodontal mechanical therapy did not affect on COPD [127].

Finally, several systematic reviews have found that about 40% of pneumonia cases are preventable by mechanical oral hygiene and local chemical disinfection with antiseptics or antibiotics [122,128].

I.6.5. Periodontitis and Adverse Pregnancy Outcomes

Pregnancy is a physiological process that sometimes has adverse outcomes including low birth weight (<2500 g), pre-term birth (<37 weeks), growth restriction or pre-eclampsia, a dangerous complication associated with hypertension and proteinuria [129]. Pre-term birth is a leading cause of newborn deaths and the second most common cause of death worldwide in children younger than five years [102,130]. Also, more or less 50% of premature births have an unknown cause, which encourages further research [131]. The potential link between the presence of periodontitis in pregnant women and adverse pregnancy outcomes has been the focus of scientific literature since these are generally associated with elevated local and systemic inflammatory markers [129,132].

It is well-known that pregnancy may amplify periodontitis progression. Gingival inflammation, along with PPD and BOP, increases during pregnancy even when plaque levels are maintained [133]. Besides, different periodontal bacteria, especially *F. nucleatum*, but also others such as *P. gingivalis* have been found in the amniotic fluid and the placenta of pregnant women with periodontitis [82,134].

There is no consensus in the literature on whether periodontitis is a risk factor for preterm birth or not. Some cross-sectional studies have shown the association between PPD and prematurity and low birth weight [135-138]. On the other hand, other papers did not find any relationship between both conditions [139-141]. Regarding the mechanisms, it is argued that inflammatory mediators released as a consequence of periodontitis could contribute to pregnancy complications. Also, periodontopathogenic bacteria or antigens present in parts of those bacteria (for instance, the cell wall) could enter the bloodstream and trigger an exacerbated response leading to a premature birth [142].

Most of the more recent meta-analysis concluded that periodontitis could foster adverse pregnancy outcomes. The OR for preterm birth and low weight in mothers with periodontitis varied from 3 to 3.57 [143,144]. Besides, three meta-analyses have identified a connection between periodontitis and pre-eclampsia, noting that periodontal women had a 2 to the 4-fold increased risk of suffering it [145-147].

At present, there is conflicting evidence to support that periodontal treatment reduces the incidence of adverse pregnancy outcomes; however, at least, literature agrees that the treatment is safe for mother and child [72,148,149]. The problem is that many studies did not include confounding factors for preterm birth, or used an ineffective periodontal treatment. In this sense, when well-conducted trials were carried out, the periodontal treatment appears to be effective (e.g. in a randomised clinical trial with 400 women, the preterm rate was 1.8% in the treatment group [200 women] *versus* 10.1% in the control group [200 women]) [149,150].

I.6.6. Periodontitis and Alzheimer's Disease

Alzheimer's is a neurodegenerative disease that is becoming a serious health problem among the elderly [83]. The incidence of Alzheimer significantly increases with age, reaching almost 50% in subjects aged 85 years [151]. This disease is usually classified into early-onset (generally associated with genetics) or late-onset (most

common and is thought to be caused by genetics and environmental factors) [83].

One of the hypotheses explaining the pathogenesis of Alzheimer's disease, gaining strength over the years, is the inflammatory theory: a chronic state of cerebral inflammation could lead to a neurodegenerative process [152]. In this context, periodontitis could be a risk factor for Alzheimer's disease by two possible pathways:

1. Inflammatory mediators released by the "low-grade systemic inflammation" induced by periodontitis are capable of entering cerebral regions and activate microglial cells, causing neuronal damage.

2. Periodontopathogenic bacteria enter the brain through the bloodstream or peripheral nerves, inducing inflammation in the central nervous system, and neuronal damage [83].

This last hypothesis is supported by some studies that found oral *Treponemas* in the trigeminal ganglia [153,154]. Lately, fungi have also been detected in the brain of corpses from patients affected with Alzheimer's, although the origin of those fungal cells is unknown [155].

On the other hand, Alzheimer's supposes a risk factor for periodontitis, since cognitive impairment leads to a lack of manual skills necessary for proper oral hygiene [156]. Although there is emerging evidence linking both diseases, there are still no intervention studies in humans that strongly support this association.

I.7. MICROBIOLOGY OF PERIODONTAL DISEASES

As we have already mentioned, periodontal diseases are multifactorial diseases that require the interaction of subgingival bacteria and the host immune-response for its initiation and progression [157].

For more than a hundred years, the complexity of periodontal microbiota has been a research focus, from the culturing of the first oral samples in the traditional microscopes to the development of pyrosequencing techniques, which have enlightened the path of future discoveries.

One of the earliest references to the study of oral microbiome dates back to 1684 when Anthony van Leeuwenhoek reported the discovery of oral flora. His statements were: "the number of these animalcules in the scurf of a man's teeth is so many that I believe they exceed the number of men in a kingdom". Two hundred years later, Robert Koch, who would later be known as the father of Modern Bacteriology, began to use nutrients in order to grow and isolate bacteria, starting the era of bacterial culture.

The culture of microorganisms was considered as the gold standard for more than a century as it was the only possible way to study bacteria. Although it has been recognised that over a half of oral bacteria have not yet been cultured or validly named [158] the culturing methods used in those first studies identified the major groups of microorganisms forming the oral microbiome, such as *Streptococcus species, Neisseria species, Veillonella species, and Fusobacterium species* [159]. However, the culturing technique has some notable disadvantages such as the cost-benefits and time-requiring, which makes it unsuitable for high-throughput screening [157]. Another drawback is the small number of oral bacteria that can be cultured by standard techniques when compared to those identified by DNA approaches [160]. In order to overcome the limitations mentioned above bacterial cultures, various molecular techniques like DNA hybridisation and polymerase chain reaction (PCR), among others, were developed [161].

Socransky et al. [162] revolutionised the pathogenesis of periodontal diseases when, in 1998, used genomic hybridisation techniques to describe different bacterial "complexes" representing bacterial associations. Thus, instead of a single pathogen causing periodontitis, like in common infectious disease, Sockranski et al. presented several "complexes" of microorganisms acting together [163], probably synergistically. Three species were described as strongly associated with the severity of disease*: P. gingivalis, Treponema denticola* and *T. forsythia*. This bacterial triad constituted the so-called "Red Complex" and was highly associated with severe chronic periodontitis. In turn, these bacteria were related to the "Orange Complex", constituted by those bacteria associated with late colonisation of dental plaque: *P. intermedia, F. nucleatum, Prevotella nigrescens, Campylobacter rectus, Peptostreptococcus micros (Parvimonas micra), Campylobacter gracilis, Campylobacter showae, Streptococcus constellatus, Eubacterium nodatum, Fusobacterium periodonticum* [162]. On the other hand, the "Yellow Complex", including species such as *Streptococcus gordoni* and *Streptococcus mitis*, or the "Green Complex" comprised predominantly by *Capnocytophaga species*, were mainly related to health or early colonisers of dental plaque.

PCR technology represents a faster and more sensitive detection of periodontal bacteria compared to traditional culture. PCR is easy to use; it can amplify small amounts of bacterial nucleic acids and detects as few as ten microorganisms in a plaque sample. Quantitative PCR or real-time PCR (qPCR) is a modified technology of the conventional PCR that not only can detect bacteria but also quantify the amplification product obtained from a sample [164]. In a meta-analysis conducted by Atieh [164], qPCR was highly associated with the accurate detection of *A. actinomycetemcomitans* and *P. gingivalis*.

There is little evidence in the literature about the development of predictive models for the diagnosis of periodontitis based on the quantification of subgingival pathobionts by qPCR [165]. However, recently, some bacterial cluster-based models with good predictive accuracy have been described. The most predictive models had an area

under the curve (AUC) with values >0.76 (sensitivity and specificity >75%). The ones with the lowest number of microorganisms were those formed by *P. intermedia*, *T. forsythia* and *F. nucleatum* or by *T. denticola*, *P. intermedia*, *T.forsythia* and *A. actinomycetemcomitans.* Distinct clusters formed by species with a different etiopathogenic role (belonging to different Socransky's complexes) had good predictive accuracy for distinguishing a site with periodontal destruction in a periodontal patient [165].

The gene coding for the 16S ribosomal RNA gene (16S rRNA) is present in all prokaryotic microorganisms, and is highly conserved, allowing its amplification by PCR. The 30S subunit of the ribosome is composed of the 16S rRNA and 21 proteins. Through the use of universal primers, it is possible to amplify, sequence and compare to public databases, describing the species present in a given sample even if they have not been previously identified [163]. Using 16S rRNA technology, followed by cloning and Sanger sequencing, numerous studies have revealed the microorganisms associated with periodontitis. These sequence analyses of the 16S gene have allowed identifying new species (even if they have not been previously cultured) [166]. Some of these recently found microorganisms are *Filifactor alocis, Megasphaera species, Peptostreptococcus species, Prevotella denticola, Eubacterium saphenum, Desulfobulbus species, Porphyromonas endodontalis* and *Cryptobacterium curtum* (Figure 5).

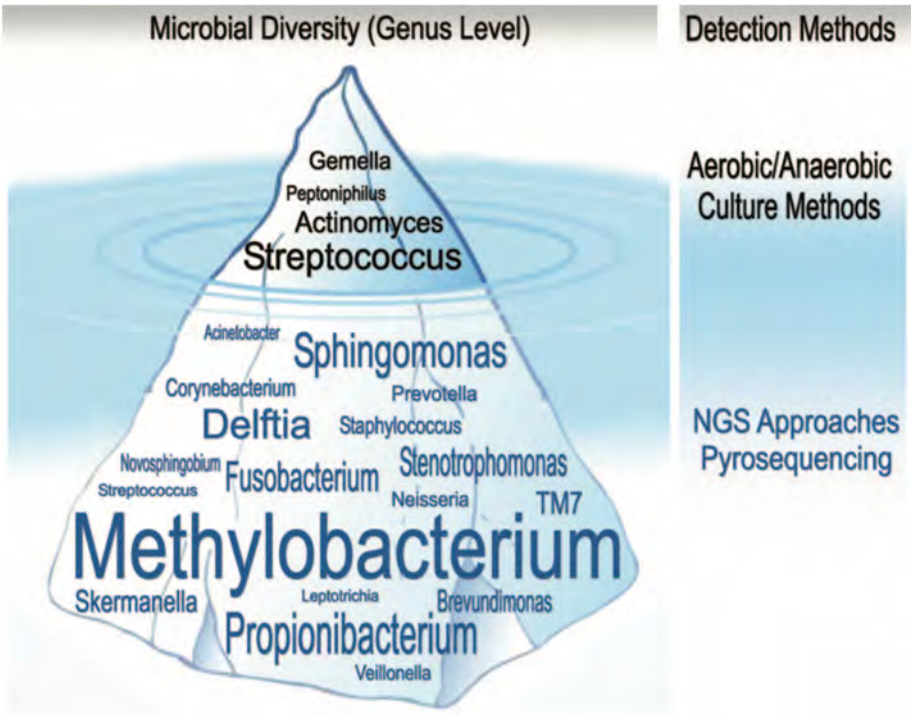

Figure 5. Example of bacterial diversity (at the genus taxonomic level) detected in blood samples after dental extractions by different approaches. Culture-based methods detected those genera at the top of the iceberg (black labelled). Pyrosequencing detected those genera at the submerged part of the iceberg (blue labelled). Font size is associated with the frequency of the bacteria determined by both methods. The image was taken from Benítez-Páez et al. [160]. Creative Commons license.

The arrival of DNA-based molecular methods and later the development of high-throughput sequencing methods like pyrosequencing and Illumina sequencing implied the development of a new field of research: metagenomics. The objective of metagenomics is, to study whole microbial communities through the DNA of their members without the need for culture [167]. The application of high-throughput sequencing (HTS) to RNA samples has allowed the study of all active microbes and expressed genes at a given time point, which has been named as metatranscriptomics [168].

The Human Microbiome Project (HMP), carried out between 2008-2012 with the aim of a comprehensive examination of human microbial diversity, used metagenomics techniques to analyse the microbiome associated with health in different sites of the body, including several sites within the oral cavity [169]. The large number of genomic results derived from the HMP confirmed the concept of "the massive bacterial colonisation of the human being", and established the beginning of a new paradigm in Microbiology. From a functional point of view, the microbiome can be considered as a human organ [169].

An essential application of HTS has been the massive sequencing of the 16S rRNA gene, which has allowed the sequencing of thousands of copies of the gene per sample, compared to the tens or hundreds by the Sanger technology. Thus, the HTS of the 16S rRNA gene, together with the metagenomics and metatranscriptomics analysis of periodontal samples, have considerably improved our understanding of the pathogenesis of periodontitis. The typical model presented by Socranski where Red Complex bacteria (*T. forsythia, P. gingivalis* and *T. denticola*) was thought to be the causative agent of periodontitis has been evolving to a much more complex paradigm. This model considers periodontitis as a dysbiosis instead of an infectious disease.

An important discovery has been the identification of these red-complex species in the healthy gingival sulcus [170], although at low levels. This fact implies that the periodontal pathogens do not infect and are already present under healthy conditions. It was then confirmed that periodontitis is initiated by a synergy and dysbiosis (i.e. an imbalance) of the microbial community [171]. This dysbiotic change of the subgingival microbiota supposes a significant change in the relative abundance of the individual members of the community, including not only to the "established" periodontopathogens, but also to a considerable number of other unknown taxa until the moment, such as *Anaeroglobus, Bulleidia, Desulfobulbus, Filifactor, Mogibacterium*, or even the uncultured *TM7 phylum* [159].

It is an exciting result that the diversity of the microbial population linked to periodontitis seems to be more extensive than previously expected, and higher than under healthy conditions [170]. This finding can be interpreted as an impaired local immune response or a higher availability of nutrients and niches [159].

In a recent systematic review of 41 articles, the authors considered that there was enough evidence in the literature to confirm the potential association with periodontitis of 17 microorganisms from the phyla: *Bacteroidetes, Candidatus Saccharibacteria, Firmicutes, Proteobacteria, Spirochaetes, Synergistetes* and even members of the *Archaea* domain [172].

On the other hand, the discovery of a high detection of periodontopathogens in low abundance in gingival health suggested that periodontitis cannot be considered as a "classic" infection, nor the bacteria involved considered pathogenic agents of infectious nature. These bacteria have been recently identified as "pathobionts" [173]. Nevertheless, there are specific strains of *A. actinomycetemcomitans* that are extremely virulent and able to transmit aggressive periodontitis, and therefore the possibility of infection with specific aggressive strains cannot be dismissed [174].

Periodontitis is characterised therefore, by a bacterial community with greater diversity, different composition and structure than oral health. The origin of the dysbiosis is, however, less understood. There are, of course, some genetic factors, and a few SNPs have been associated with a higher risk of periodontitis. A second, well-established factor is the accumulation of plaque, which could initiate an inflammatory response by the host, leading to an environment rich in GCF, tissue remnants and proteins. This scenario could favour proteolytic bacteria such as the periodontal pathogens, which are therefore considered "inflamophylic" [171]. This fact would, in turn, increase the proportions of the pathogens, further inducing inflammation in a feedback loop (Figure 6).

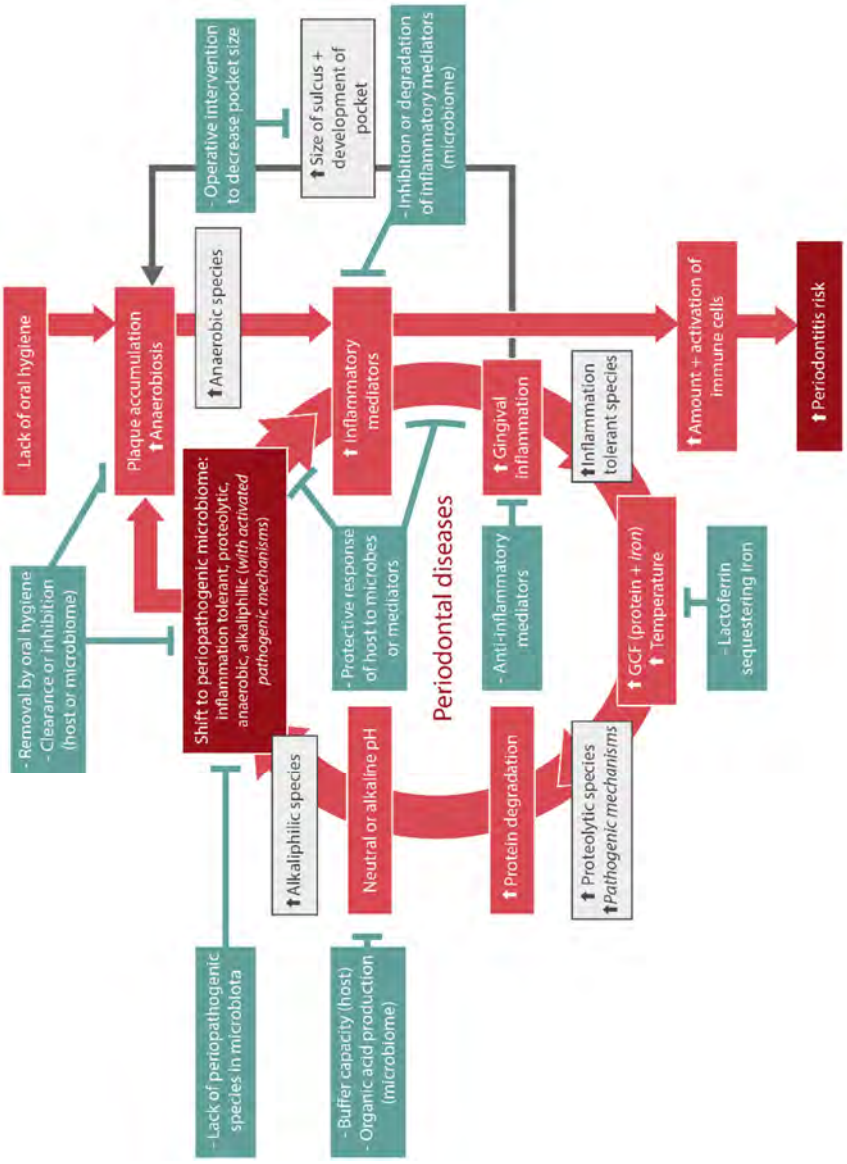

Figure 6. Positive feedback loop leading to periodontal diseases. In this positive feedback loop, the disease trigger capable of causing a shift toward a periopathogenic microbiota is the plaque accumulation. The grey arrow represents another chain of self-reinforcing events (e.g. the formation of a pocket allows for more plaque accumulation). In the green boxes are the mechanisms that could prevent various stages of the loop. Some of these mechanisms are likely to contribute to resilience. Italicised font indicates possible involvement. The image was taken from Rosier et al. [18]. Creative Commons license.

The confirmation of these findings by several articles [170,175], has led the scientific community to the rethinking of the main characteristics of oral polymicrobial diseases (such as periodontitis, gingivitis, halitosis and caries); equally, those affecting other areas of the body, such as bacterial vaginosis or rhinosinusitis [175]. From a clinical point of view, this new etiopathogenic characterisation casts doubts on the convenience of the preventive or therapeutic approach of polymicrobial oral diseases. This approach is based on antimicrobial treatments [176], whose application is questionable given that periodontal pathogens are present under healthy conditions and are very unlikely to be eradicated. Instead, a new tendency in preventive approaches has been proposed, in which the principal purpose would be to re-establish the homeostasis between the oral microbiota and the host [159,177,178]. This fact could be achieved, for example, by the use of prebiotics and probiotics that could restore the balance and favour the growth of beneficial bacteria. Similarly, strategies that reduce plaque accumulation and promote host defences without the elimination of the whole microbial community [18].

The results derived from the first phase of the HMP [169] and later works [170,175] revealed that the taxonomic composition of the microbiomes between the subjects differed significantly. This finding indicates that doing exclusively taxonomic characterisation is not enough to reveal the relationships between the microbiome and specific states of health or disease. In some first approximations made with the HMP, the analysis of the biological properties of the microbiome, such as the community of transcripts, proteins or metabolites, implied losses or gains of critical functions of the microbiome associated with specific diseases. The enormous importance of evaluating the biological functionality of human microbiomes justifies the implementation of the second phase of the HMP (Project of the Integrated Human Microbiome or IHMP [179]). In this second phase, the biological role of the microbiome in health and disease through three models with different human conditions is studied [179]. Recent metatranscriptomic studies with periodontal samples have also been relevant, as they indicated the species that appear to be active under disease conditions, as well as the metabolic pathways that are highly expressed under disease conditions

[180]. For example, the expression of gingipains and other virulence factors from *P. gingivalis* have confirmed that this bacterium could act as a "keystone pathogen". This observation means that this microorganism could initiate the inflammatory feedback loop by impairing the immune response and facilitate the outgrowth of other pathogens, giving rise to the dysbiosis [181].

Shortly, the simultaneous metagenomic and metatranscriptomic analysis of periodontitis and the study of microbial interactions in health/disease under the focus of metabolic modelling will allow deepening into the pathogenesis of this polymicrobial dysbiosis. It will also clarify the potential of the oral microbiome (and specific species) as a diagnostic/prognostic indicator of periodontitis, and contribute to the search for new treatment modalities.

I.8. IMMUNOLOGY: HOST RESPONSE IN PERIODONTAL DISEASES

Once bacteria and their products activate the acute phase of inflammation, there is an imbalance between the microbiota and the host immune system. Inflammation is considered a protective mechanism. Through an inflammatory cascade, mast cells are activated and begin to release TNFalpha, resulting in, among other things, an increase in vascular permeability and an increase in polymorphonuclear neutrophils (PMNs); PMNs release lysosomal enzymes that are responsible for tissue degradation. An invasion of lymphocytes and macrophages occurs in the periodontal tissues that cause even more significant degradation of the tissue collagen. However, this phase is still reversible since bone destruction has not occurred yet [84,182].

Host-susceptibility is crucial in the development of periodontitis. In susceptible individuals, the acute phase of inflammation is not resolved, because the elimination of inflammatory cells, leukocytes and neutrophils, does not occur. The inflammation becomes chronic, resulting in the periodontal tissues breakdown through the osteoclast activation, and therefore, periodontitis (Figure 7) [84,182].

When acute inflammation persists, macrophages and dendritic cells process and present bacterial antigens to the adaptive immune system.

Naïve T helper lymphocytes interact then with the antigens presented differing into various subsets of T lymphocytes: Th1, Th2 and Th17, depending on the cytokines that they are going to produce (Table 5) [84,182].

Table 5. Immune system and cytokines.

Immunity	Cytokines
Th1	IL2, IL3 and IFNgamma
Th2	IL1, IL3, IL4, IL5, IL6, IL10, IL13, TNFalpha and GMCSF
Th17	IL17

Moreover, fibroblasts from the gingival connective tissue are also involved in inflammation by producing cytokines, prostaglandins (PGEs) and proteolytic enzymes. These inflammatory factors are capable of affecting the progression of periodontitis due to some cytokines such as IL1beta, IL6 and IL17 (except IL17E), granulocyte-macrophage colony-stimulating factor (GMCSF) and TNFalpha that are among the most critical pro-inflammatory mediators reported stimulating osteoclast activation [182]. Anti-inflammatory cytokines, on the other hand, play a significant role in the regulation of T-cell subsets acting at many levels. Some of these mediators, such as IL13 and interferon (IFN) gamma, have an inhibitory effect on the osteoclastogenesis [183].

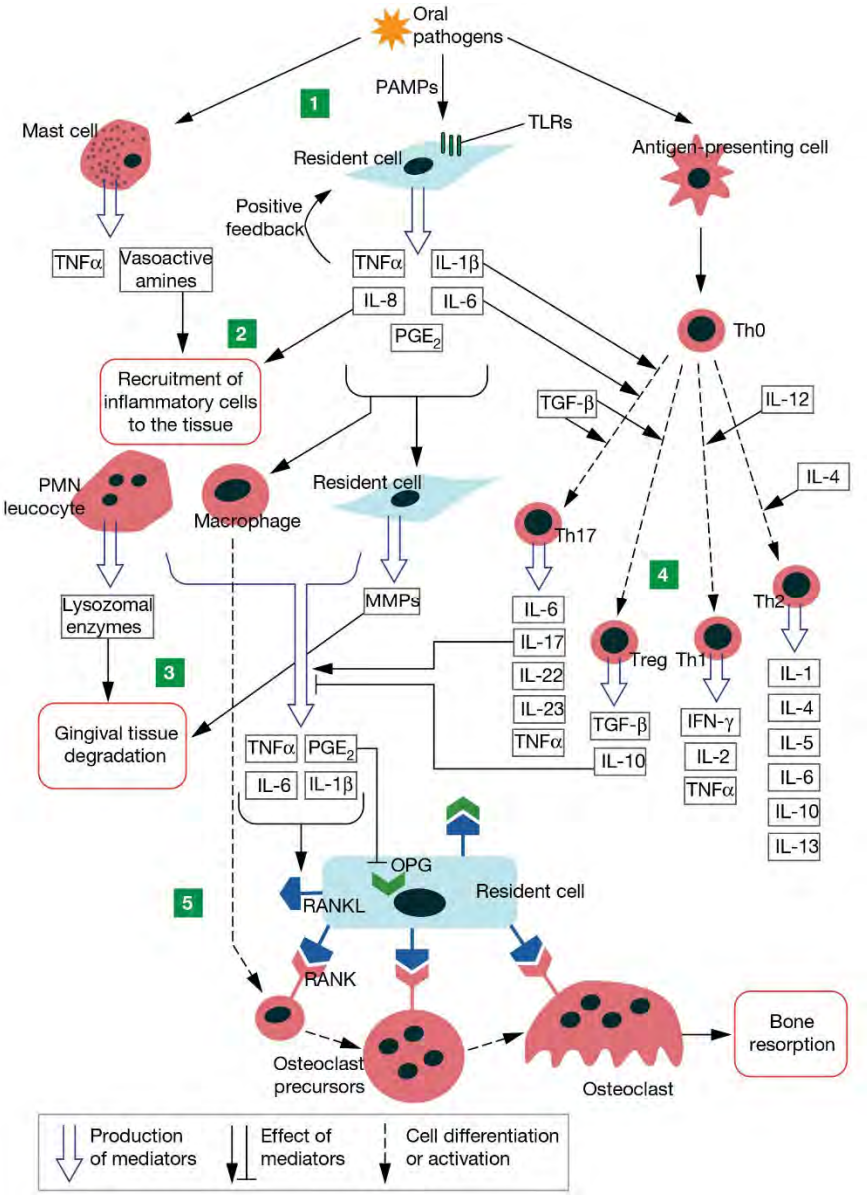

Figure 7. Inflammatory mediators in the pathogenesis of periodontitis. The image was taken from Yucel-Lindberg and Båge [182]. Creative Commons license.

1.8.1. Type of Samples: GCF, Saliva and Serum

Collecting samples at the site of the disease provides large amounts of information, and because cytokines are locally produced in periodontal tissues. The analysis of cytokine levels in the GCF is considered as an accurate measure to diagnose the level of inflammation [184]. Besides, several authors have referred to the importance of GCF to evaluate periodontitis since it is the "mediating element" between the bacterial plaque adhered to the tooth and the periodontal tissues [163,185].

GCF is a local serum exudate of the gingival connective tissue that provides a great deal of information about host-bacteria interactions occurring in the gingival sulcus [163]. GCF consists of a combination of substances from serum, connective tissues and subgingival plaque: host inflammatory markers, enzymes, oral bacteria and leukocytes, among others [184].

In healthy periodontium, GCF is the transudate produced by the osmotic gradient in the gingival sulcus. Under normal conditions, GCF has a relatively small volume, but its amount raises as the severity of the disease increases [186].

Although GCF recollection is a relatively non-invasive method, it is time-consuming, and it has to be collected by a trained professional [185-187]. Typically, GCF is collected by the insertion of paper strips during an adequate time, usually 30 seconds, to get enough fluid. All those points contaminated with blood or dental plaque should be discarded [186]. In addition to paper strips, there are other methods to collect fluid, like micropipettes or the Periotron, an instrument designed to quantify fluid volumes [184,186].

On the other hand, saliva is considered as the body's mirror. It has been proposed as an attractive diagnostic fluid of the periodontitis progression because of its easy, abundant and non-invasive collection [188,189]. The composition of saliva is a mixture from the salivary glands, GCF, serum, expectorated secretions, bacteria and different types of human cells [190]. There are two main methods of collecting

saliva: unstimulated and stimulated. For diagnostic purposes, the most typical is non-stimulated whole saliva, collecting it by passive drivelling [190].

Although there is evidence that suggests that the principal source of cytokines in the saliva is GCF, the dilution of GCF containing these cytokines in saliva may explain the lack of consensus in the literature. Some studies suggest that salivary biomarkers could discriminate between health and disease [191-193], while others could not find differences [194,195]. The exception seems to be IL1beta since, in most studies, it is elevated in periodontal patients [191,192,196,197]. Concerning matrix metalloproteinases (MMPs), MMP8 has been considered as a promising biomarker of periodontal disease. A meta-analysis published in 2018, concluded that MMP8 salivary levels were significantly elevated in periodontitis patients compared to healthy controls [198].

Besides, considering the periodontitis-systemic diseases link, cytokine levels in serum have also been proposed in order to detect "low-grade systemic inflammation" [96,189,199], but the results are inconclusive yet [200,201]. Only the CRP has enough evidence to support that it is elevated in periodontitis patients comparing to controls and gingivitis [96]. An interesting fact is that elevated CRP levels are a known risk factor for CVDs [202]. Furthermore, it has been shown that SRP mildly decreases CRP serum levels [96,203].

I.8.2. Methodological Techniques for Detecting Biomarkers

Accurate and sensitive methods for detecting biomarkers are essential characteristics for the study of cytokines and its involvement in different pathogenesis [204]. There are different techniques to measure these biomarkers in periodontal diseases, although immunoassays are usually chosen because they are biomarker-specific, easy to carry out and standardised [205]. As enzyme-linked immunosorbent assay (ELISA) was developed in the 1970s, during many years, ELISA was the standard for quantitative analysis of

cytokines and other inflammatory biomarkers [206]. Since then, the cytokine assays have been evolving.

Evidence highlights that cytokines work in networks, so being able to detect and quantify multiple cytokines in just a sample is a demanding prerequisite [204,207]. The appearing of new techniques as the multiplex immunoassays has revolutionised Experimental Biology [208]. In our field, ELISA is the most widely used technique, although the tendency these last few years seem to be that multiplexing techniques are replacing ELISA [209]. Bioassays techniques, on the other hand, are capable of determining active concentrations of cytokines [204]. From the standard immunoassays and in order to overpass their traditional limitations, many techniques have been developed: immunochromatographic methods, capillary electrophoretic immunoaffinity chromatography or flow-based assay using fluorescent-labelled antibodies, among others. There are other approaches to measure cytokines, instead of immunoassays, as mass spectrometry, capable of detecting peptides instead of whole proteins.

Mass cytometry is another technique but limited by the available fluorophores. Besides, DNA detection started to be used in the detection of biomolecules. The qPCR allows determining gene expression for cytokine regulation. Finally, aptamers have been widely used as molecular recognition thanks to their capability to bind many analytes (Figure 8) [204].

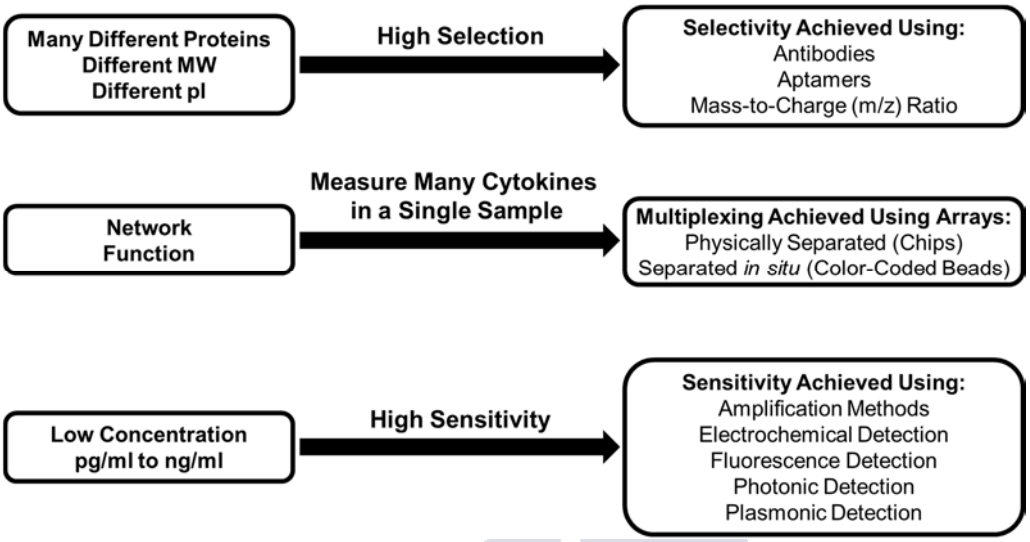

Figure 8. Methods to detect biomarkers. The image was modified from Stenken and Poschenrieder [204] with permission of Elsevier.

I.8.2.1. ELISA

ELISA is a traditional immunoassay technique in which an antigen is detected by an antibody bound to an enzyme capable of generating a colour change to identify a substance.

The antigen-antibody binding is performed after adding the sampling containing specific antibodies. The washing step ensures the elimination of all the unbound material. If the antibody is bound to the antigen, it is detected by the addition of an enzyme-labelled "conjugate." Again, all unbound material is removed. Finally, the substrate is added. If in the previous step the conjugate is bound, the substrate is transformed by the action of the enzyme contained in the conjugate causing a colour change. The intensity of the colour of this reaction is supposed to be proportional to the activity of the antibody in the sample [208,210].

ELISA-based assays have several main disadvantages: they only measure one analyte per time; they require around 50-100 microliters of sample, which can substantially increase the price and limit the

number of essays with a given sample volume; they depend excessively on the operator skills; may be influenced by room temperature; and they have a narrow dynamic range, which compels to dilute cytokine concentrations above the range. Also, ELISA techniques present another disadvantage related explicitly to GCF; the volumes obtained from the fluid are so small that mixing samples from different sites is a requirement [206,208,211,212].

Besides all these, ELISA platforms have two critical advantages: their kits are widely available, and they are capable of differentiating active and inactive forms of cytokines [204].

There are different types of ELISA techniques: direct ELISA, which we have previously described, indirect ELISA, sandwich ELISA, which is used to detect antigens and competitive binding ELISA (Figure 9) [210].

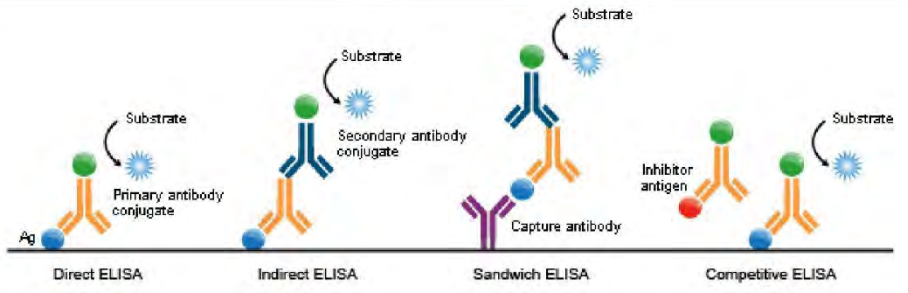

Figure 9. ELISA techniques. The image was taken from Wikimedia [213]. Creative Commons license.

In a 2016 review, the authors claimed that not all commercial kits of ELISA provide the same quality-control of data, so the comparative results of different studies have to be interpreted with caution [209].

I.8.2.2. Multiplex Bead Immunoassay

A multiplex assay is a type of assay capable of measure multiple analytes simultaneously. Luminex technology combines fluorescence detection, precision microspheres and covalent conjugation of antibodies to the microspheres to quantify up to 100 analytes at the same time [214].

Although the concept of multiplex assays exists since the late 1990s, it has been recently introduced to the general use due to the appearance of the relatively low-cost instruments [206,208].

Luminex is an assay that modifies the spectral properties of the polystyrene beads or microspheres that contains to distinguish the different analytes. Meanwhile, it simultaneously measures the amount of fluorescence associated with phycoerythrin (PE)-conjugated streptavidin, reported as mean fluorescence intensity (MFI) (Figure 10). As a result, up to one hundred detection reactions can be carried out at the same time using minimal sample volumes, resulting in a more efficient diagnosis tool [206,208,210].

Polystyrene beads are read on a dual-laser flow-based detection instrument, such as the Luminex® 100™, Luminex 200™ or Bio-Rad®Bio-Plex® analyser. One laser classifies the bead and determines the analyte that is being detected. The second laser determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound.

In addition to the Luminex 100, Luminex 200 or Bio-Rad Bio-Plex dual-laser, flow-based analysers, magnetic beads can be read using the Luminex MAGPIX® analyser. A magnet in the MAGPIX analyser captures and holds the magnetic beads in a monolayer, while two spectrally distinct light-emitting diodes (LEDs) illuminate the beads. One LED identifies the analyte that is being detected and, the second LED determines the magnitude of the PE-derived signal. Each well is imaged with a CCD camera.

I.8.2.3. Cytometric Bead Array

The cytometric bead array (CBA) system is a multiplexed bead assay from BD Biosciences (San Jose, CA, USA) [206]. Analytes are measured by detecting different fluorescent intensities and multiplex analysis (Figure 10) [200,206]. The fundamental advantage of the CBA over Luminex is that it can be used in a flow cytometer already installed in laboratories [206,215].

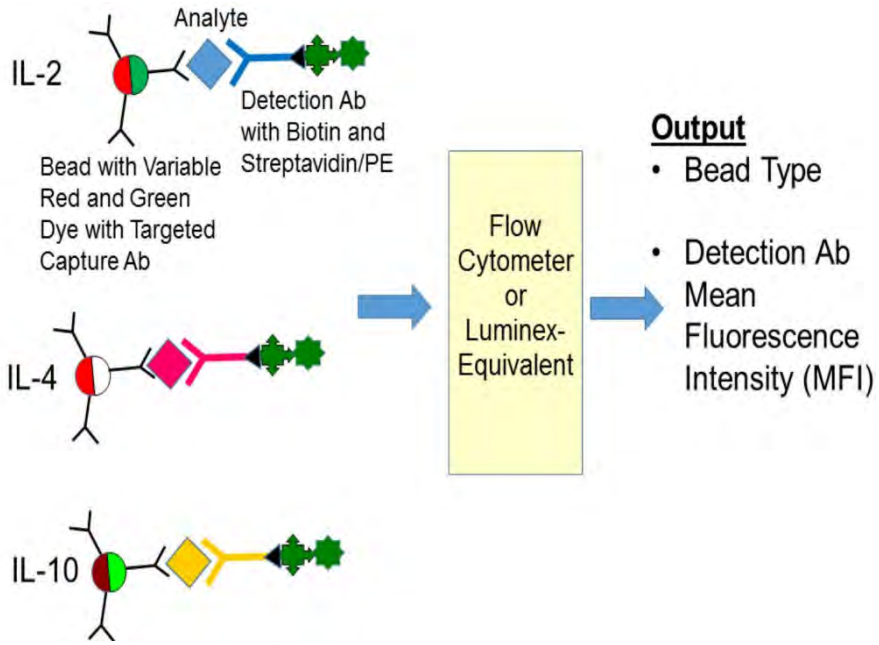

Figure 10. Overview of bead-based immunoassays. Different colour-coded beads with dyes that fluoresce either red or green are used. The instrument measures the bead colour intensity and the mean fluorescence intensity of the labelled detection antibody, which is typically labelled with PE-conjugated streptavidin. The image was taken from Stenken and Poschenrieder [204] with permission of Elsevier.

In order to elucidate how ELISA and Luminex could correlate, several authors have conducted different studies comparing both assays. Elshal and McCoy [206] and Khan et al. [216] showed a good correlation between the biomarkers tested, although the degree of correlation varied amply. DuPont et al. [217], analysing nine cytokines (IL1, IL4, IL5, IL6, IL10, IL12p70, IL13, IFNgamma and TNFalpha)

in phlebotomy samples, observed excellent correlations between ELISA and Luminex for seven of the nine cytokines. These authors found an acceptable correlation for IL13, but it was IL12 that showed different results between both assays. Furthermore, according to Richens et al. [215], Luminex results not only correlates with ELISA's but also its kits are reliable and reproducible.

In general, multiparametric technology has the same or better sensitivity than the traditional ELISA techniques, requires less processing time and cost and allows the analysis of multiple analytes simultaneously in small volume samples [208,212,216,218].

In this Thesis, we have applied a Luminex technique to compare healthy and periodontal oral samples from controls and periodontitis patients.

I.8.3. Biomarkers of Periodontal Diseases: Cytokines

As we previously described before, periodontitis implies a complex interaction microbiota-host immune-response. This host immune-response is characterised by infiltration of the gingival tissues by neutrophils, macrophages and lymphocytes, and the generation of high concentrations of destructive mediators [219]. These mediators not only act as initiators and regulators of the immune response, but they also mediate in the tissue damage, which leads to functional loss and clinical disease [220,221]. The extension of this immune-reaction would determine the progression and severity of periodontitis [209].

Cytokines (in greek "Cyto-", cell, and "-Kinos", movement) are low-molecular-weight soluble proteins, with an essential role in homeostasis [222]. Cytokines are involved in the initiation and progression of immune-inflammatory processes, and they are produced by different cell types. In the acute phase of inflammation, cytokines are released by epithelial cells, fibroblasts and phagocytes; while in adaptive immunity, they are released by lymphocytes (Table 6) [223,224]. The cytokine production in the organism is extremely regulated, and in healthy people, their concentrations are measured in

picomolar/ml [204]. Higher concentrations of cytokines are associated with inflammation and disease progression [204].

Table 6. Pro-inflammatory cytokines, source cells and target cells [184,199,225,226].

Cytokines	Source Cells	Target Cells
IL1	macrophages, monocytes, lymphocytes, endothelial cells	T and B lymphocytes
IL6	B and T lymphocytes, macrophages, fibroblasts, endothelial cells, keratinocytes	B and T lymphocytes, other cells
IL12	macrophages, B lymphocytes	Th1 lymphocytes, NK cells
IL17	activated T lymphocytes	multiple
GMCSF	T lymphocytes, macrophages, endothelial cells, fibroblasts	macrophages, granulocytes
TNFalpha	monocytes/macrophages, lymphocytes, neutrophils	multiple

In this Thesis, we decided to focus on the principal cytokines studied in recent times concerning the pathogenesis of periodontitis. To make it easier to understand this complex topic, we have decided to divide them generally into pro-inflammatory and anti-inflammatory cytokines, assuming that some of them may have both effects depending on the functions they carry out [227]. Besides, cytokines act as a network, so different cytokines carry out the same functions, allowing that in the absence of one specific cytokine another with a similar activity would take its place; consequently, the response would continue to be activated by another pathway. This vital mechanism is called biological redundancy [207,228].

I.8.3.1. Pro-inflammatory Cytokines

There are different families of cytokines, but given the inflammatory nature of periodontitis, most cytokines investigated concerning its pathogenesis are pro-inflammatory. Of all these cytokines, IL1beta is the most studied, followed by TNFalpha and IL6 [222].

I.8.3.1.1. Interleukin 1 alpha

IL1alpha is a pro-inflammatory cytokine that belongs to the IL1 cytokine family, mainly produced by macrophages (Figure 11, left). Already in the early ’90s, the potent effect over bone metabolism of the IL1 was known, so its role in the pathogenesis of periodontal diseases has been widely described [229,230]. IL1alpha regulates immune and inflammatory responses and hematopoiesis.

IL1alpha can be detected in GCF samples, independently of the gingival or periodontal status [231-233]. Concerning the cytokine’s concentrations, various authors agreed to affirm that periodontal patients showed significantly higher levels of IL1alpha in GCF compared to healthy controls [232,233] and gingivitis patients [234]. Thunell et al. [231] analysed 22 mediators in GCF in six chronic periodontitis patients using the Luminex instrument. Of these 22 biomarkers, only IL1alpha and IL1beta appeared significantly higher in disease sites when compared with healthy sites in the same patients. Although the sample size of the study was quite small gives us an idea of the potential importance of this cytokine [231].

Besides, according to the literature, IL1alpha concentrations diminished in smokers periodontitis patients comparing non-smokers, which would explain the low levels of clinical inflammation in the last ones [233,235,236].

Although IL1alpha has been detected in saliva, its levels in periodontitis were not different from those of healthy patients (Table 7) [237,238]. As far as we know, there is no evidence of IL1alpha in serum.

I.8.3.1.2. Interleukin 1 beta

IL1beta is a pro-inflammatory cytokine with a major role in the pathogenesis of the periodontal diseases (Figure 11, right).

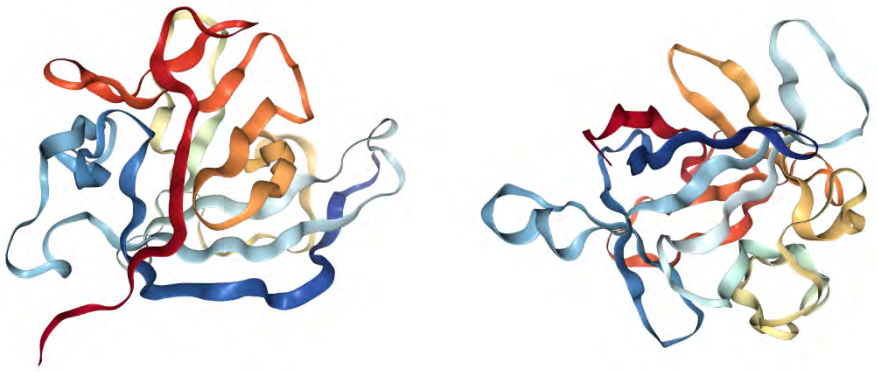

Figure 11. Solution structures of left, IL1alpha [239] and right, IL1beta [240]. The images were taken from RCSB [241]. PDB IDs: left, 2KKI; right, 9ILB [242]. Images free of copyright.

IL1beta is mainly produced by PMNs, but also by other cells like lymphocytes and endothelial cells. IL1beta increases bone resorption by activating osteoclasts, prevents bone regeneration and degrades extracellular matrix (connective tissue) by MMPs. On the other hand, IL1beta also induces the production of PGE2. IL1beta is the most studied cytokine in the literature and was the first cytokine to be analysed in the GCF [243].

In the literature, IL1beta has been detected in all GCF samples so far [232,244,245]. As same as IL1alpha, there is evidence in the literature confirming higher levels of IL1beta in GCF from periodontal patients compared to healthy controls [232,233,246-248]. IL1beta also appears to be significantly increased in GCF from periodontitis comparing to gingivitis [234,244]. Although the correlation between the expression of inflammatory mediators in gingival biopsies and GCF may not be accurate [249], Górska et al. [201] analysed the cytokine profiles in the inflamed gingival tissue and found that IL1beta levels were significantly higher.

Even, it has been detected higher concentrations of IL1beta in deep sites compared to shallow sites in patients with both types of periodontitis (generalised aggressive and chronic), and it seems to recognise active diseased sites [199,233,244,250]. Ertugrul et al. [251] and Giannopoulou et al. [252] also found higher amounts of IL1beta in aggressive periodontitis comparing chronic periodontitis. However, these findings must be taken with caution due to Ertugrul et al. [251] also recognised that they collected higher volumes of GCF in aggressive periodontitis and Giannopoulou et al. [252] did not mention the volumes. Besides, other authors suggested that people with locally elevated IL1beta were more likely to suffer from periodontitis [253-255].

The relationship between IL1beta and tobacco has been quite studied, although the results are inconclusive. There is some evidence that shows that IL1beta decreases in smokers patients [256], but in most of the series, tobacco did not affect IL1beta expression [257-259].

Several investigations have identified elevated levels of IL1beta in saliva from periodontitis patients compared to healthy individuals [191,192,196,197,258,260]. Nevertheless, Teles et al. [194] could not find any significant difference between IL1beta levels in periodontitis or control patients. Although these authors used Luminex, one possible explanation of their results would be the mild grade of the periodontitis compared with other studies [194]. Two exhaustive reviews concluded that IL1beta is a reliable analyte in periodontitis studies (Table 7) [190,209].

There is controversy with IL1beta levels in serum, due to the results are inconclusive. Increased levels in periodontitis have been reported by some authors [201,246] but others, however, could not observe significant differences between periodontal individuals and controls [200,261].

IL1beta has been correlated in different studies with various cytokines, such as IL1ra, IL11 and IL6 [247,248]. Besides, IL1beta has been correlated in multiple times with CAL and PPD

[201,232,244,247,262-265]. The correlation between IL1beta and BOP and plaque index (PI)/ bacterial plaque levels (BPL) remains unclear due to some studies revealed positive associations [251,263,266], but others not [232,247].

I.8.3.1.3. Interleukin 6

IL6 is a pro-inflammatory cytokine produced by various types of cells such as monocytes, T and B cells, fibroblasts and osteoblasts, among others (Figure 12, left) [199]. IL6 increases acute inflammation promotes the evolution of a chronic inflammatory state and can stimulate osteoclast differentiation and inhibit bone formation, leading bone remodelling [267].

About the frequency of detection of this cytokine, IL6 could be detected in most of the GCF samples tested [232,245,268].

IL6 has also been widely studied in the literature, but the results are contradictory. In some studies, healthy sites with periodontitis showed significantly higher amounts of IL6 than healthy controls [232,233,247]. On the contrary, Teles et al. [269] did not detect significant increment of IL6 levels in GCF associated with generalised aggressive periodontitis. Even, Khalaf et al. [270], reported a significant reduction (35%) of levels of IL6 in patients suffering from periodontitis compared to healthy controls.

IL6 has been related to those sites with increased periodontal destruction and therefore linked to the severity of the disease [233,245,252,271].

IL6 have also been identified in saliva, but unlike IL1beta, the results were inconclusive (Table 7). Ebersole et al. [192,272], using Luminex technique, found increased levels of IL6 in saliva from periodontal patients. In another series, Prakasam and Srinivasan [273] detected a higher concentration of IL6 in chronic periodontitis patients, using the ELISA technique. Another study compared healthy, chronic and aggressive periodontitis and found that IL6 levels were increased in both periodontitis groups [274]. Teles et al. [194] also used Luminex,

but they concluded that IL6 could not discriminate between periodontal patients and healthy individuals. Although the severity of the disease could be the reason of the different results, Rathnayake et al. [196] who analysed 451 subjects (healthy individuals, moderate and severe periodontitis patients) using both Luminex and ELISA, came to the same conclusion that Teles et al. [194]. Scannapieco et al. [275] neither found differences between groups. Interestingly, in the study carried out by Khalaf et al. [270], using ELISA, IL6 levels decreased significantly in periodontal patients compared to controls.

In serum, the role of IL6 has been found controversial. De Queiroz et al. [200] and Shimada et al. [276] found that IL6 increased levels in the test group, although the results of de Queiroz were not significant [200]. The findings published by Gümüs et al. [274] agreed with the previous authors; they detected higher levels of IL6 in aggressive and chronic periodontitis comparing healthy controls. On the contrary, Robati et al. [277] showed that IL6 was elevated in serum from patients with aggressive periodontitis. A plausible explanation for these results could be the clinical differences between patients due to IL6 has been linked to the severity of the disease.

In 2011, Tymkiw et al. [233] analysed the influence of smoking in 22 cytokines, including IL6, concluding that IL6 expression was diminished in GCF of smokers periodontitis patients compared to non-smokers. Like with IL1alpha, this would explain why smoker patients have less clinical signs of inflammation than non-smokers.

IL6 has been positively correlated with other inflammatory biomarkers such as IL1beta, OSM (a gp 130 cytokine), IFNgamma, IL4 and IL12 [245,247,271]. It has also been negatively associated with IL10 and IL2 [271]. Although some studies found positive associations between IL6 and the clinical parameters CAL, PPD and BPL [247,264,271], others did not find any [232,278]. Fujita et al. [264] also observed a weak correlation (Spearman 0.42) with BOP, although Zhang et al. could not find any [268].

I.8.3.1.4. Interleukin 12 subunit p40

IL12p40 is a pro-inflammatory cytokine, usually known as a component of the cytokines IL12p70 and IL23 (Figure 12, right).

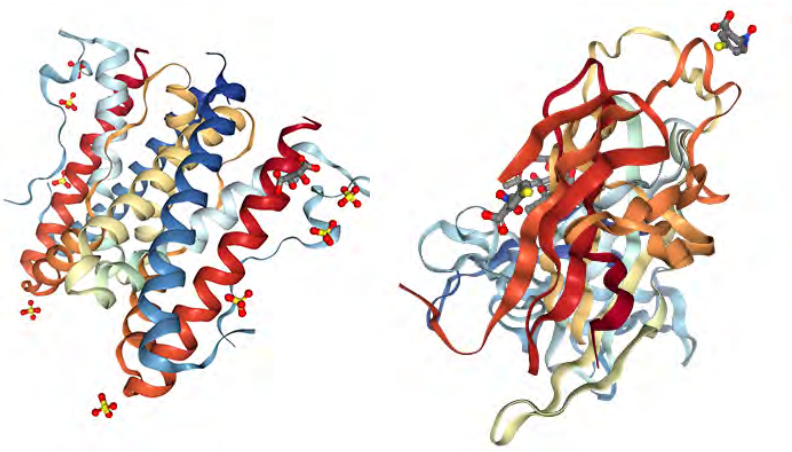

Figure 12. Crystal structures of left, IL6 [279] and right, IL12p40 [280]. The images were taken from RCSB [241]. PDB IDs: left, 1ALU; right, 1F42 [242]. Images free of copyright.

IL12p40 attracts macrophages and promotes Th1 cells development, regulating the balance between Th1 and Th2. It stimulates natural killers (NK) cells and T cells to produce other cytokines such as IFNgamma [281,282].

Concerning the frequency of detection of IL12p40, Orozco and coworkers [234] did not detect it in the GCF samples from patients with chronic periodontitis applying different techniques (ELISA and Luminex). Thunell et al. [231] and Tymkiw et al. [233] got the same results in their respective series using Luminex.

We have not been able to find much information in the literature about this cytokine, neither in GCF nor saliva or serum, and the conclusions were unclear (Table 7). Tymkiw et al., in his 2011 article [233], compared GCF from healthy and diseased subjects using Luminex, and they found significantly higher amounts of IL12p40 in the last ones. In another study, the levels of IL12 were also elevated in

chronic periodontitis subjects [282], while in the paper carried out by Shimada et al. [266] there was no significant difference between healthy and disease sites. Sánchez-Hernández and coworkers [283] analysed gingival tissues from aggressive and chronic periodontitis patients, detecting increased levels of IL12p40 in the first ones. By contrast, Orozco et al. [234], studied it in GCF using ELISA, but there were no differences between gingivitis or periodontitis sites.

We could only found one study analysing IL12 levels in saliva and three papers in serum. In saliva, cytokine levels seemed to be higher in periodontitis, but more studies are needed to confirm it [284]. In serum, the results available were as well inconsistent. One study described only low levels of IL12p40, while the findings of others were contradictory. Sánchez-Hernández et al. [283] obtained increased levels in aggressive and chronic periodontitis compared to controls; while Cairo et al. [285] did not detect differences between these two types of periodontitis.

Tymkiw et al. [233] studied the relationship of IL12 with tobacco. They concluded that smoking produces an immunosuppressive effect in some pro-inflammatory cytokines, such as IL12p40.

Indeed, the same investigation group found that healthy sites with periodontitis showed significantly higher amounts of IL12p40 than healthy controls. Another publication, however, did not find correlations between clinical parameters and salivary levels of IL12 [284].

I.8.3.1.5. Interleukin 17 family – IL17A and IL17F

IL17A and IL17F are pro-inflammatory cytokines belonging to the IL17 cytokine family and Th17 (Figure 13). During the last years, it has been more studied its relation with periodontitis since Th17 stimulates osteoclastogenesis [286].

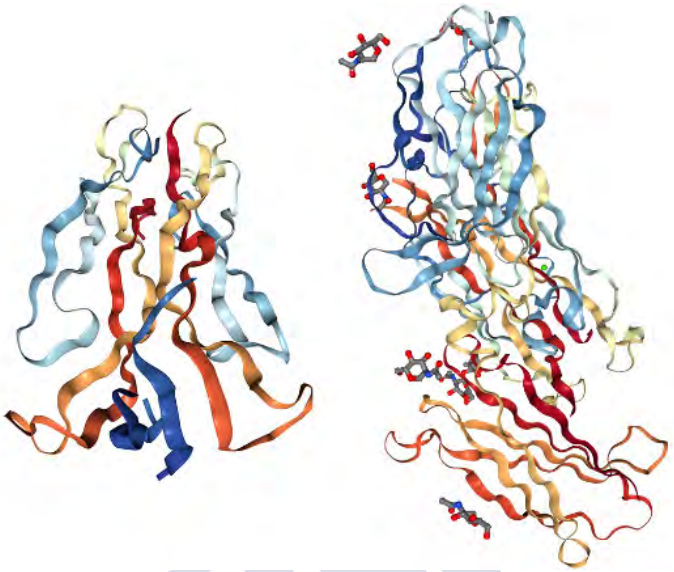

Figure 13. Crystal structures of left, IL17A [287] and right, IL17F receptor complex [288]. The images were taken from RCSB [241]. PDB IDs: left, 4HR9; right, 3JVF [242]. Images free of copyright.

IL17A's main function is to amplify inflammatory responses. It also induces the production of other cytokines such as IL1beta, IL6, GMCSF, TNFalpha and chemokines (IL8). IL17F has a similar pro-inflammatory function to IL17 and may contribute to host defence and autoimmune function of Th17 cells [286,289].

About the concentration of IL17 in GCF, Shaker and Ghallab [290] and Mitani et al. [291] agreed that IL17 was increased in periodontitis patients. Shaker and Ghallab [290] also found increased levels of IL17 in GCF from aggressive periodontitis compared with chronic periodontitis, suggesting a potential role in the pathogenesis of periodontal diseases.

Another study, applying an ELISA technique, revealed that GCF IL17's concentration was significantly lower in periodontal patients with deep pockets (≥5 mm) than those periodontal patients with shallow pockets (≥4 mm) and healthy controls [292]. On the contrary, in the

Awang et al. 's series [193], GCF IL17A levels were higher in patients with chronic periodontitis and correlated positively with clinical parameters. Also, Vernal et al. [293] found higher total amounts of IL17 both in GCF and tissue supernatants of chronic periodontitis patients compared with healthy controls.

However, only a few studies have evaluated salivary levels of IL17. Whereas Awang et al. [193] found that IL17A levels in saliva were higher in periodontitis than in healthy subjects, others did not find any differences between periodontal patients and controls (Table 8) [195,273,294]. In serum, increased levels of IL17 have been detected in aggressive and chronic periodontitis, demonstrating that these subjects have elevated systemic levels of this pro-inflammatory cytokine [193,295].

IL17A showed positive correlations with IL17E, IL17F and the cytokine ratio IL17A/F [193,296]. Also, one paper described a tendency toward a negative correlation with IL35, but this was not statistically significant [291]. For its part, IL17F has been positively correlated with IL17E and the IL17A/IL17F ratio [296].

It should also be highlighted the positive correlation between the IL17 family and the clinical parameters CAL, PPD and BOP in several studies [193,291]. Besides, Awang et al. [193] also associated the IL17A/IL17F ratio with CAL, PPD and BOP.

I.8.3.1.6. Granulocyte-macrophage colony-stimulating factor

GMCSF is a pro-inflammatory cytokine, which is part of the inflammatory cascade. GMCSF stimulates stem cells to produce granulocytes and monocytes (Figure 14, left) [297].

In the literature, studies investigating the role of GMCSF in periodontal diseases were few [233,269]. This cytokine showed higher amounts in diseased sites not only in chronic but also in aggressive periodontitis compared to healthy subjects in two studies [157,233]

while in another study, the authors could not find differences between disease and healthy sites [231].

Furthermore, Tymkiw et al. [233] analysed GCF samples of 52 subjects using Luminex and comparing healthy and diseased locations from periodontitis patients showed higher concentrations of GMCSF in the latter.

The only article in saliva to our knowledge concluded that GMCSF salivary levels could not discriminate between healthy and periodontal patients [194]. Likewise, differences between periodontitis and controls in serum were not significant in the only paper we could find [200].

I.8.3.1.7. Tumour necrosis factor alpha

TNFalpha is a pleiotropic inflammatory cytokine produced by many different cells in the periodontium (Figure 14, right). It has multiple functions; it is associated with bone reabsorption and stimulation of apoptosis of fibroblasts and inhibition of bone collagen synthesis, causing limited repair of the periodontal tissues [84]. TNFalpha also increases levels of MMPs, PGEs and receptor–activator of nuclear factor-κB ligand (RANKL) and regulates IL1beta and IL6 production [84,220,298]. In periodontitis, TNFalpha is involved at an early stage in the inflammatory cascade due to its release from mast cells in response to bacteria [182].

Tymkiw et al. [233], applying a Luminex technique, did not detect TNFalpha in the GCF samples from patients with chronic periodontitis. However, one study found that the frequency of detection of TNFalpha was higher in the gingival tissue supernatants from periodontal patients than in those from healthy controls, and another one found TNFalpha in more than 85% of the periodontal samples [201,268].

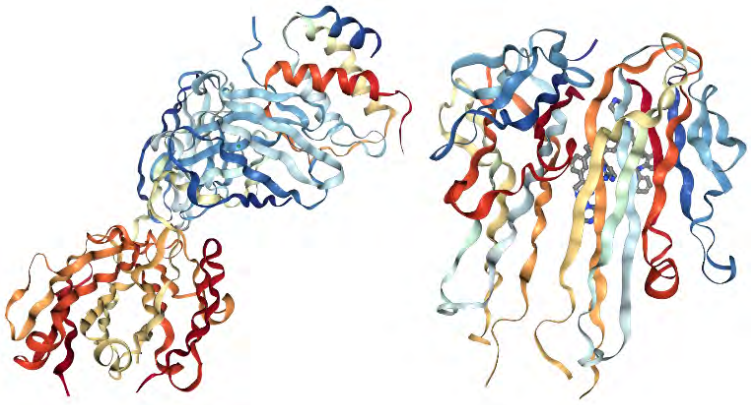

Figure 14. Crystal structures of left, GMCSF [299] and right, TNFalpha in complex with a small molecule [300]. The images were taken from RCSB [241]. PDB IDs: left, 6BFQ; right, 5MU8 [242]. Images free of copyright.

The evidence about TNFalpha in the literature is contradictory. Several authors detected significantly higher levels of this cytokine in the untreated disease sites in comparison to the control non-disease sites, both in the gingival tissue and GCF samples from chronic periodontitis and healthy controls [201,232,301,302].

However, different studies demonstrated no significant differences between healthy and diseased GCF sites [264,266,303,304]. Other authors observed that periodontitis subjects (aggressive and chronic) had significantly lower levels of TNFalpha [269,301], while Kurtis et al. [302] found that total TNFalpha were statistically higher in aggressive periodontitis than in healthy subjects. Additionally, another study found higher total amounts of TNFalpha in aggressive compared to chronic periodontitis [251].

The effect of smoking on GCF levels of TNFalpha remains unclear. While César-Neto and colleagues [236] observed that TNFalpha levels decreased in smokers periodontitis patients compared to non-smokers; Boström et al. [305] found no difference between them.

TNFalpha was correlated with MCP1, suggesting a mechanism of amplification [302]. Furthermore, there is enough evidence in the literature to correlate TNFalpha with clinical parameters CAL, PPD, BOP and PI [201,251,264,268,302].

There is some controversy over the role of TNFalpha in saliva (Table 7). Some authors obtained this cytokine elevated in saliva, being able to discriminate between periodontitis and healthy subjects, although not between aggressive and chronic periodontitis [274,306]. Other studies, however, did not find differences between them or even found TNFalpha reduced in periodontal patients [194,301,307]. Therefore, TNFalpha can not be considered as an optimal salivary biomarker of periodontitis yet [209].

Nevertheless, the results of this cytokine in serum appeared to be more consistent. TNFalpha was significantly elevated in chronic periodontitis patients compared to healthy controls [200,201,274,295,308,309]. In two publications, levels of TNFalpha in aggressive and chronic periodontitis could not discriminate between these two types of periodontal disease [274,285].

Table 7. Pro-inflammatory cytokines in GCF and saliva.

Cytokine	In GCF	In Saliva
IL1alpha [209,222,250]	Increases	Similar
IL1beta [209,222,250]	Increases	Increases
IL6 [209,222,250]	Increases	Unclear
IL12(p40) [222]	Unclear	No evidence
IL17 [209,222]	Increases	Unclear
TNFalpha [209,222]	Unclear	Unclear

Conclusions derived from literature reviews.

I.8.3.2. Anti-inflammatory Cytokines

The anti-inflammatory cytokines control the proinflammatory cytokine response (Table 8). Contrary to the pro-inflammatory cytokines, only a few papers have focused on the role of anti-inflammatory mediators, being the most researched IL4 and IL10 [222].

Table 8. Anti-inflammatory cytokines, source cells and target cells [184,199,225,226].

Cytokines	Source Cells	Target Cells
IL2	Th0, Th1 lymphocytes	T and B lymphocytes
IL3	T lymphocytes, mast cells	hematopoietic stem cells
IL4	Th2 lymphocytes, macrophages, basophils	B lymphocytes, Th1 lymphocytes, macrophages
IL5	Th2 lymphocytes macrophages	eosinophils, lymphocytes
IL10	Th2 lymphocytes	B and T lymphocytes
IL12p70	macrophages, B lymphocytes	Th1 lymphocytes, NK cells
IL13	activated T lymphocytes	B lymphocytes, monocytes
IFNgamma	Th1 lymphocytes, leukocytes	multiple

I.8.3.2.1. Interleukin 2

IL2 is an anti-inflammatory cytokine that has a crucial role in cell immunity (Figure 15). IL2 belongs to Th1 immunity and promotes the differentiation and activation of T cells, helping to fight against the infection, and acts together with IL12 to increase NK cytotoxic activity. Indeed, IL2 promotes the production of other cytokines such as TNFalpha, GMCSF and IFNgamma [310].

In general, the frequencies of detection of the anti-inflammatory cytokines in GCF samples from periodontal patients are generally lower. Górska et al. [201] observed that the frequency of detection of IL2 showed a higher percentage in the diseased tissues, while Rescala et al. [244] detected it in 57% of cases.

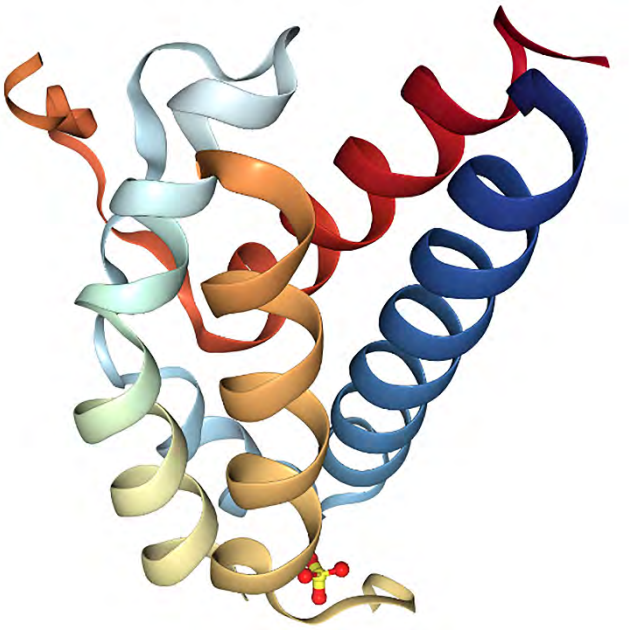

Figure 15. Crystal structure of IL2 [311]. The image was taken from RCSB [241]. PDB ID: 1M47 [242]. Image free of copyright.

Numerous authors, analysing different types of samples (GCF and gingival tissue), demonstrated that the IL2 amounts were significantly higher in periodontal patients in contrast to healthy controls [201,233]; as well as in deep sites with respect to shallow sites in chronic periodontitis [271] or generalised aggressive periodontitis [244]. On the contrary, in the paper published by Khalaf et al. [270], results revealed that GCF IL2 levels were significantly reduced in patients suffering from severe periodontitis (around 47%).

In saliva, no differences in IL2 levels or concentrations have been reported. Two studies could not discriminate between chronic periodontitis and healthy patients analysing IL2 levels through different techniques (Luminex and ELISA) [194,270]; and in another paper, IL2 was not even detected [237]. Between healthy, chronic and aggressive periodontitis, no significant differences were neither perceived [312].

In agreement with these results, are those found in serum samples. Most of the investigations could not detect differences between healthy and periodontitis groups [200,270]. However, one study identified significantly higher concentrations in periodontitis patients while another found lower levels of IL2 in the patient's group. Authors of this study attributed their results to the participants' age, which was 35 years old for their study and 47 years in Górska's study [201,309].

Johnson and Serio [271] correlated IL2 with other cytokines: IL2 was positively associated with IL10 and IFNgamma, and negatively with IL6. In two papers, IL2 has also been linked with clinical parameters. While Rescala et al. [244] mentioned that IL2 is correlated in general with clinical parameters, Górska et al. [201] only found significant correlations with CAL. On the contrary, Johnson and Serio [271] could not find a significant association between IL2 and PPD.

I.8.3.2.2. Interleukin 3

IL3 is an anti-inflammatory cytokine produced by activated T cells and basophils. IL3 stimulates secretion and differentiation of monocytes and macrophages [313].

There is a lack of information on IL3 about periodontal diseases, either in GCF, saliva or serum. To the best of our knowledge, there is one article analysing IL3 in GCF in healthy and periodontal subjects. In this study, this cytokine was detected in low amounts in all groups of patients. IL3 from diseased sites in periodontal patients showed significantly higher amounts than in healthy controls [233]. In the same article, Tymkiw et al. [233] reported that IL3 were higher in disease sites in smokers comparing to non-smokers.

In the only article that we could find analysing this cytokine in saliva, levels were below the detection limit [237].

I.8.3.2.3. Interleukin 4

IL4 is an anti-inflammatory cytokine belonging to Th2 or humoral immunity (Figure 16, left). IL4 has an essential role in the inflammatory

response regulating macrophage function. Indeed, IL4 can inhibit the secretion of different pro-inflammatory cytokines (IL1beta, TNFalpha, IL6) and chemokines (IL8) [314].

Papathanasiou et al. [262] found detectable levels for IL4 only in the 9% of healthy sites from periodontal patients while it was not detected in periodontal sites. On the contrary, Górska et al. [201] observed that the frequency of detection of IL4 differed between both groups, showing a lower percentage in the diseased tissues.

According to Tymkiw et al. [233], IL4 levels from diseased sites in periodontal patients showed significantly higher amounts than in the healthy controls. However, Rescala et al. [244] detected that GCF IL4 levels were significantly higher in shallow sites from subjects with gingivitis compared to shallow sites from subjects with generalised chronic and aggressive periodontitis. In this sense, Pradeep et al. [315], after studying GCF IL4 levels in several groups (healthy, gingivitis and periodontitis), obtained that IL4 concentrations decreased progressively from healthy to periodontitis subjects (up to 6 times less). Salvi et al. [316] also noted the progressive decrease of IL4 in according to the periodontal deterioration. These results reinforced the hypothesis on the protective role of IL4 in the periodontium, reducing the periodontal destruction. However, Górska et al. [201] did not find significant results between groups.

Nevertheless, the findings that we could find in the literature about IL4 in saliva are inconsistent (Table 9). Teles et al. [194] could not discriminate between healthy and disease, and neither could Ramseier et al. [312]. However, another study found higher IL4 concentrations in periodontal subjects [273]. This lack of congruity is probably due to the severity of the disease, its complexity, and the inherent characteristics associated with saliva or a mixture of all of them.

In serum, four studies revealed no significant differences between periodontitis and healthy patients [200,201,295,309], while another one reported that IL4 decreased in generalised aggressive periodontitis [277].

One study found a positive correlation of IL4 with IL6 and a negative correlation with IFNgamma [271]. Regarding the possible associations of cytokine IL4 with tobacco or clinical parameters, we could not find too much information. Kamma and coworkers [257] found that GCF IL4 levels increased in smokers patients, comparing to non-smokers. Tymkiw et al. [233], a few years later, did not detect differences between smoking and non-smoking population. On the other hand, neither Papathanasiou et al. not Johnson and Serio did not obtain associations between IL4 and clinical parameters [262,271].

I.8.3.2.4. Interleukin 5

IL5 is an anti-inflammatory cytokine produced by Th2 cells (Figure 16, right). IL5's main function is the activation and regulation of eosinophils [313].

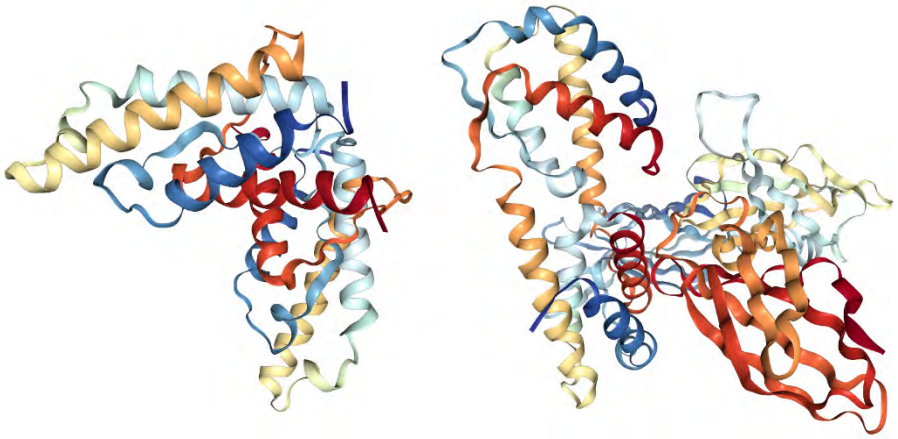

Figure 16. Crystal structures of left, IL4 [317] and right, IL5 with receptor [318]. The images were taken from RCSB [241]. PDB IDs: left, 1HIK; right, 3VA2 [242]. Images free of copyright.

Various authors have observed that the IL5 quantities in the GCF from periodontitis patients were below the detection limits of the assay [231,233]. Therefore, we have not found information to confirm whether or not GCF IL5 levels are elevated in periodontitis.

In saliva, we found two studies analysing this cytokine. As in GCF, Fine et al. [237] could not detect IL5. Teles et al. [194], however, detected this cytokine in saliva, although it could not distinguish between health and disease.

Two other papers in serum agreed with the above statements. Neither de Queiroz et al. [200] nor Andrukhov et al. [309] nor reported any significant differences when comparing IL5 levels in periodontal patients and healthy patients.

I.8.3.2.5. Interleukin 10

IL10 is an anti-inflammatory cytokine belonging to Th2/humoral immunity (Figure 17, left). IL10 can inhibit LPS and other cytokines, including TNFalpha, IL1beta, IL12, IFNgamma and GMCSF. This cytokine has an essential role in maintaining the health and stability of periodontal tissues inhibiting pro-inflammatory cytokines and so, preventing bone resorption [319,320].

Some authors that studied IL10 in GCF observed that the quantities of the marker were undetectable by the assay [231,233]. On the contrary, Górska et al. [201] detected IL10 in 70% of samples from healthy controls.

There is heterogeneity among the studies published in the literature. Reis et al. [232] found significantly higher GCF IL10 levels in the untreated periodontal sites about control non-disease sites. On the contrary, Górska et al. [201] detected similar IL10 levels in gingival tissue biopsies between health and chronic periodontitis. Even, Teles et al. [269], after expressing the data for the cytokines as a percentage of the total level of cytokines, obtained that aggressive periodontitis subjects had a significantly lower percentage of IL10 (34.6% ± 17.2% *versus* 56.6% ± 12.1% in the periodontally healthy subjects). These results are in agreement with those published by Casarin et al. [321] who detected lower levels in aggressive periodontitis than in chronic periodontitis.

In saliva, data from different studies were also inconsistent (Table 9). Teles et al. [194], as well as Ramseier et al. [312] using different techniques (Luminex and ELISA, respectively), found no differences between IL10 salivary levels in chronic periodontitis *versus* healthy patients. Others authors, however, detected lower levels in chronic and aggressive periodontitis comparing healthy controls [273,274].

This heterogeneity of results in clinical studies continues in serum. Two studies revealed significantly increased levels in serum from periodontitis than in controls, one using ELISA and the other a CBA [201,309]. On the contrary, other series, also using ELISA, demonstrated lower serum levels of IL10 in diseased samples comparing healthy [274,308]. However, two more papers, using Luminex, described similar IL10 levels between healthy and disease patients [200] and between chronic and aggressive periodontitis [285].

Johnson and Serio [271] positively correlated IL10 with IL2 and negatively with IL6. Some authors studied the correlation between IL10 with PPD and CAL and also with tobacco [232,236,264,268]. Although Fujita et al. [264] found a weak association between IL10 and BOP, another two studies did not find any [232,268]. Regarding the relationship with smoking, César-Neto et al. [236] found that GCF levels of IL10 were decreased in smokers periodontitis patients compared to non-smokers.

I.8.3.2.6. Interleukin 12 subunit p70

IL12p70 is a cytokine formed by IL12p40 and p30 subunits (Figure 17, right) [322].

IL12 is a cytokine with both pro- and anti-inflammatory activity [234,323,324]. IL12p70 is produced by macrophages, monocytes and neutrophils, and it has a regulatory function [325]. IL12 contributes to the Th1 immune response, and it is an inducer of INFgamma production by NK cells and T cells. Due to IFNgamma itself can also activate IL12 production, there is positive feedback between both cytokines. LPS of periodontopathogens are also inducers of IL12. Nevertheless, IL4

inhibits the production of IL12 by inducing Th1 cells to turn to Th2 cells [322].

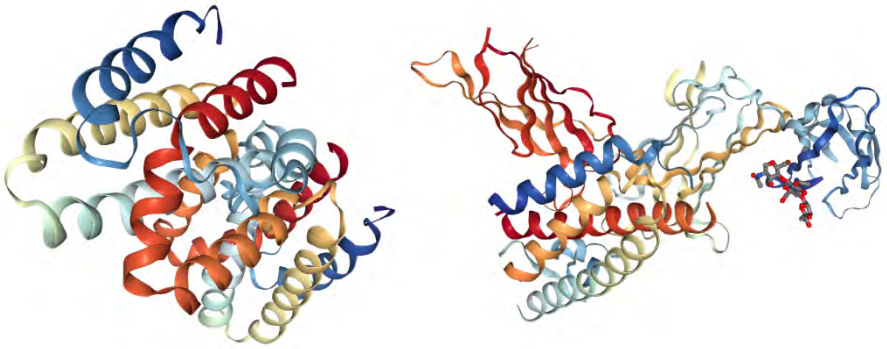

Figure 17. Crystal structures of left, IL10 [326] and right, IL12p70 [280]. The images were taken RCSB [241]. PDB IDs: left, 1VLK; right, 1F45 [242]. Images free of copyright.

Various authors in their respective series observed that the GCF quantities of IL12p70 from periodontal patients were non-detectable because their levels were below the range of the assay [231,233,234]. We found only two articles analysing this cytokine in GCF with contradictory results and none in saliva. Johnson and Serio [271] demonstrated that IL12 was significantly lower in diseased gingival biopsies than in the healthier ones. Tsai et al. [325], however, found an increased total amount of this cytokine in disease sites in periodontitis comparing gingivitis and healthy patients.

In serum, we could only find two articles studying IL12p70 in aggressive and chronic periodontitis patients. In both series, the authors stated that there were no statistically significant differences between both groups [277,285]. In one study, IL12 was positively correlated with IL6 and negatively correlated with IL18 and sulcus depth [271].

I.8.3.2.7. Interleukin 13

IL13 is a pleiotropic and anti-inflammatory cytokine with an essential role in the inflammatory response (Figure 18). IL13 belongs to Th2 immunity and has similar effects on immune cells to IL4 [327].

Indeed, IL13 modulates the production of IL1, TNFalpha, IL8 and acts synergistically with IL2 to regulate IFNgamma synthesis [328].

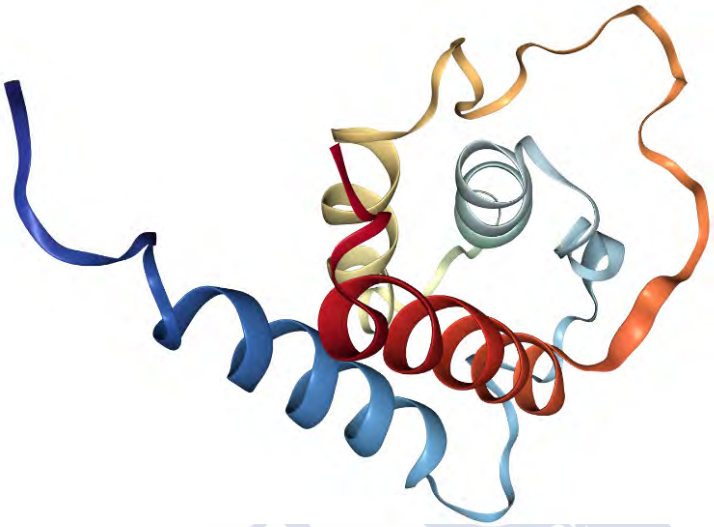

Figure 18. Crystal structure of IL13 [329]. The image was taken from RCSB [241]. PDB ID: 1GA3 [242]. Image free of copyright.

Quantities of IL13 from periodontal subjects could not be noticed in various studies because the levels were out the range of the assay [231,233].

Concerning the concentration of IL13 in GCF, Teles et al. [269] reported a trend, although not statistically significant, of IL13 elevated in aggressive periodontitis. In saliva, two studies detected no differences between chronic periodontitis, gingivitis and healthy groups [312] or between aggressive periodontitis and healthy controls [237]. Gonzales et al. [330] also found similar IL13 serum levels in aggressive periodontitis and controls.

I.8.3.2.8. Interferon gamma

IFNgamma is a soluble cytokine produced by lymphocytes and NK cells that belongs to the Th1 or cellular immunity. IFNgamma can carry out both anti-inflammatory and pro-inflammatory functions: it is a

potent activator of macrophages, increases osteoclastogenesis and promotes the differentiation of Th0 lymphocytes into Th1, among other actions [222,331].

Górska et al. [201] observed that the frequency of detection of IFNgamma in subgingival biopsies showed no differences between healthy controls and periodontitis patients.

Concerning the IFNgamma levels, several authors, analysing different types of samples (GCF and gingival tissue), demonstrated that the concentrations were significantly higher in periodontal patients when compared with healthy controls [201,233]. Papathanasiou et al. [262] found significantly higher levels of IFNgamma in GCF from periodontal sites comparing to healthy sites in patients with periodontitis. Also, these authors observed a statistically significant decrease in this anti-cytokine in healthy sites of patients with chronic periodontitis as compared to gingivitis sites in patients with gingivitis [262]. These findings could indicate that IFNgamma levels depend on the inflammatory status of the site and not on the disease status of the subject [262]. On the contrary, the literature did not evidence an association between GCF IFNgamma levels and generalised aggressive periodontitis nor between these levels and the periodontal status of the sampled site [244,269,321]. In a recent systematic review of the last ten years, IFNgamma was significantly higher in the GCF of the periodontitis group [332].

Regarding the role of IFNgamma in saliva from periodontitis patients, no differences between health and disease were found in the literature [275], or levels were undetectable [312] (Table 9). Scannapieco et al. [275] carried out a longitudinal study for five years in patients with chronic periodontitis and healthy patients, concluding that there was no association between salivary levels of IFNgamma with the alveolar bone loss. Also, in salivary samples from aggressive periodontitis and control patients, the levels of IFNgamma remained similar between groups [194,237].

However, findings relating to IFNgamma in serum showed conflicting results. Górska et al. [201] reported higher concentrations of this cytokine in periodontal patients than in controls. Also, Andrukhov et al. [309] found that gamma IFN levels were 5.5 times higher in the serum of periodontal patients compared to healthy subjects. On the contrary, de Queiroz et al. [200] detected IFNgamma levels reduced in periodontitis, although it was not statistically significant. Duarte at al. [295] described no significant differences in concentrations or levels of IFNgamma between healthy controls, chronic and aggressive periodontitis. Cairo et al.'s [285] results were in agreement with this, due to they did not observe differences between the two types of periodontal disease.

Johnson and Serio [271] positively correlated IFNgamma from gingival biopsies with IL6 and IL2. They also correlated IFNgamma negatively with IL4, IL18 and with sulcus depth. Dutzan et al. [333] analysed and followed up 106 chronic periodontitis patients during two months to conclude that IFNgamma levels were significantly higher in active locations than in the inactive ones. On the other hand, Górska et al. [201] found a positive correlation between IFNgamma levels and clinical parameters CAL and BOP.

Table 9. Anti-inflammatory cytokines in GCF and saliva.

Cytokine	In GCF	In Saliva
IL4 [209,222]	Unclear	Unclear
IL10 [209,222]	Unclear	Unclear
IFNgamma [222]	Increases	No evidence

Conclusions derived from literature reviews.

I.8.3.3. Effect of Periodontal Treatment over Cytokines (in GCF)

Periodontitis consists of a homeostasis failure caused by an exaggerated inflammatory response. Therefore, it seems logical to suppose that the return of the tissue's balance and the absence of inflammation would also have effects on the cytokine's profile [223].

In order to clarify the effects of non-surgical treatment (SRP) in cytokines in chronic periodontitis patients, several authors conducted systematic reviews and meta-analysis [222,334-336]. Stadler and coworkers [222] included for their meta-analysis data of 10 mediators from 57 studies (26 cross-sectional and 31 single-arm/case-series). Only two inflammatory markers, IL1beta and IL17, showed substantial evidence to support that they decrease after treatment. However, IL4 increased after it, and others, such as IFNgamma, IL10, TNFalpha or IL6 remained similar.

Nascimiento et al. [335] carried out a meta-analysis to investigate the effect of periodontal treatment on clinical and immunological parameters in obese subjects, which included only three studies. Nascimiento et al. [335] agreed with Stadler's meta-analysis that non-surgical treatment improved periodontal signs and symptoms of periodontitis in obese subjects, but they did not find enough evidence to assure a reduction in GCF markers [335].

Eshghipour et al. [336] carried out a systematic review to assess the impact of smoking on the cytokine profile in periodontitis patients. They included 13 cytokines/chemokines from nine studies, but the results must be taken with caution due to only TNFalpha was assessed in two studies, whereas the remain cytokines only in one. In four of the included studies, five mediators were decreased in the smoker periodontitis group after periodontal treatment. However, they concluded that the evidence was not strong enough to prove that periodontal treatment affects cytokines in smokers periodontal patients [336].

Kellesarian and coworkers [334] performed a systematic review analysing the efficacy of periodontal treatment alone compared with antimicrobial photodynamic therapy. Six studies were finally included in the review. Interestingly, these authors [334] observed that pro-inflammatory mediators (IL1beta, TGFbeta, MMP8) seemed to decrease more in periodontitis patients that underwent SRP with antimicrobial photodynamic therapy than in patients only treated with SRP; consequently, the combination of therapies is more effective than

the mechanical debridement alone [334]. These data are mainly in agreement with the conclusions of a previous meta-analysis that compared the clinical outcome after different non-surgical treatments of chronic periodontitis [73,74]. In the first meta-analysis, SRP alone was correlated with a 0.5 mm of gain in CAL. Various adjunctive therapies (systemic antimicrobials, chlorhexidine chips, photodynamic therapy with a diode laser, and local antimicrobials, among others) were more effective in both meta-analyses than SRP alone [73,74]. In the meta-analysis of 2015, the average of CAL improvements over SRP was 0.2-0.6 mm and had a moderate level of evidence [74]. In the previous one, CAL improvement was over 0.310 mm and the PPD reduction was 0.407 mm [73].

On the other hand, de Lima Oliveira et al. [225] analysed the effect of periodontal treatment on the GCF cytokines of 24 patients with aggressive periodontitis and 25 healthy controls. They concluded that periodontal treatment might affect biomarkers by lowering IL1beta, GMCSF and increasing levels of IL10.

I.8.4. Other Biomarkers of Periodontal Diseases

Although in this Thesis we will focus mainly on cytokines, we would also highlight the importance of other types of mediators in the pathogenesis of periodontitis, describing briefly some of the most studied, and assuming that many more of them have been described in the literature. Table 10 details numerous biomarkers described in GCF and saliva, classifying them into various groups: 1) host-derived enzymes; 2) inflammatory mediators and products; 3) tissue breakdown products and 4) others [188,337].

Table 10. Oral biomarkers described in GCF and saliva [188,337].

Enzymes		Inflammatory Mediators and Host Response Modifiers	Tissue Breakdown Products	Others
BG	Alpha1-proteinase inhibitor	PGE2	Laminin	8-OHdG
MMPs	Glycosidases	CRP	Osteopontin	Albumin
TIMP1	Trypsin-like enzymes	PA	Osteocalcin	NO
AST	Immunoglobulin-degrading enzymes	PAF	Calprotectin	Melatonin
Lysozyme	Aminotransferase	SP	Fibronectin	Urate
Amylase	Arylsulfatase	Cytokines	Haemoglobin	Ascorbate
Arginase	Stromyelysins	PAI2	Chondroitin 4-sulfate	Cortisol
Lysozyme	Alanine aminopeptidase	Calgranulin A (MRP8)	Chondroitin 6-sulfate	Ca
Dipeptidyl peptidase	Beta-N-acetyl-hexosaminidase	Neopterin	ICTP	PAF
Chitinase	Neural protease	Vasoactive intestinal peptide	GAG's	HGF
ALP	Creatinine kinase	Neurokinin A	Osteonectin	Keratin
Gingipain	Myeloperoxidases	Leukotriene B4	Hyaluronic acid	Complement C3
Cathepsin G,D,B	Alpha2-macroglobulin	CD14	Hydroxyproline	Epidermal growth factor
Esterase		Cystatins	OPG	
Elastase		RANTES		
LDH		VEGF		
		Lactoferrin		
		MCP1		
		Antibacterial antibodies: IgG1, IgG2, IgG3, IgG4, IgM, IgA		

I.8.4.1. Chemokines

Chemokines are signalling proteins secreted by different cells involved in cellular chemotaxis. Chemokines play a significant role in cell trafficking, as they are responsible for attracting leukocytes to the infection site. Chemokines also have other types of functions, such as cell proliferation and apoptosis, angiogenesis or host defence [84,182].

The most studied chemokine concerning periodontitis is IL8 (also known as CXCL8). IL8 is a potent chemoattractant of PMNs with a direct effect over osteoclasts and the activation of neutrophils during inflammation [338,339]. IL8 is commonly detected in the initial stages of periodontitis and associated with subclinical inflammation [182,223]. Increased IL8 levels have been found in the GCF of patients with periodontitis and correlated with the clinical parameters and disease severity [338,340]. Also, the decrease in IL8's GCF levels after periodontal treatment has been detected [231]. However, in a meta-analysis carried out in 2017, the authors concluded that although IL8 from gingival tissues of chronic periodontitis patients were elevated compared to controls, the GCF levels of IL8 (in pg/µl) were lower in the periodontitis patients [339].

MCP1 (monocyte chemoattractant protein-1, also known as CCL2), MIP1alpha (macrophage inflammatory protein 1-alpha or CCL3) and RANTES (regulated on activation, normal T cell expressed and secreted or CCL5) have also been frequently studied and associated with raised expressions in chronic periodontitis [222]. These three chemokines are synthesised by various cell types such as fibroblasts, macrophages, endothelial cells or lymphocytes [338]. MCP1 is in charge of the chemoattraction of macrophages, which could contribute to increasing the severity of periodontitis [338]. In GCF and saliva, MCP1 increased as the severity of periodontitis does, and its levels decreased after periodontal treatment [231,233,341]. Also, Stadler et al. [222] concluded in their systematic review that the GCF levels of MCP1 were increased in perio patients compared to controls. Like MCP1, MIP1alpha is a potent chemoattractant of macrophages and has been positively related to severity, and it is considered the most abundant

chemokine in the tissue's sites of periodontitis [342,343]. On the other hand, RANTES has been found elevated in active periodontal pockets, and it was undetected in GCF from healthy subjects [338,344]. Like IL8, RANTES levels decreased after periodontal treatment [231].

I.8.4.2. Tissue and Bone Remodelling

At the beginning of the disease, during the inflammation phase, different cytokines and prostaglandins are released. When periodontitis progresses, MMPs are liberated, causing the breakdown of connective tissue. As the severity increases, so they do the levels of various cytokines, including IL1, TNFalpha and RANKL with a significant role in osteoclastogenesis, which finally triggers the alveolar bone loss [220].

PGE2, involved in several biological processes, is a stimulator of bone resorption. MMPs are involved in the degradation of the extracellular matrix, the remodelling of periodontal tissues, and the regulation of cytokines. Elevated levels of both have been strongly associated with periodontitis [220]. Cells of the periodontal ligament synthesise more copious amounts of PGEs when it is inflamed; while MMPs that comprise a family of about 25 members are secreted by neutrophils and macrophages [84,345]. As we already mentioned, MMPs are involved in collagen degradation [190]. MMP8 is the most studied metalloproteinase in periodontal disease, followed by MMP13 [345]. MMP8 has been found increased in GCF, saliva and even serum samples and associated with significant reductions after periodontal treatment [346-349]. Some authors described correlations with clinical parameters [346], while others could not find anything [347]. GCF and salivary MMP8 is, therefore, considered a reliable marker for diagnosing chronic periodontitis and monitoring the disease [198,350]. MMP13, for its part, has been detected elevated in saliva and active infection sites [348,351].

RANKL and osteoprotegerin (OPG) are critical regulators of bone remodelling; therefore, they have also been studied concerning periodontal diseases [1]. There is enough evidence to support that

increased levels of the first one, and low levels of OPG participate in bone destruction in periodontitis [182]. RANKL is synthesised by several pro-inflammatory cytokines; thus, the increase of this kind of cytokines in the periodontium can contribute to osteoclastic bone loss by affecting the RANKL/OPG ratio [1].

Transforming growth factor beta (TGFbeta) is a growth factor that has a protective role against tissue destruction in periodontal disease. TGFbeta has an immunosuppressant effect of pro-inflammatory mediators and correlates negatively with RANKL levels in periodontitis [223]. Khalaf et al. [270] found higher GCF and salivary levels of TGFbeta in periodontitis patients, and they affirmed that TGFbeta could be a potential biomarker of periodontitis.

I.8.5. Cytokines and Diagnostic Accuracy of Periodontitis

As mentioned before, clinical parameters are useful for evaluating the severity of periodontitis and the response to therapy. However, they are only partially able to determine current disease activity due to some of them, such as CAL, measure mainly past episodes of bone destruction [52,189,352]. Recently, biomarkers have been included in the new classification of periodontal disease and considered useful for staging and grading periodontitis [52]. Determining biomarkers for recognising periodontitis could contribute to its early detection.

MMP8, as we have already mentioned, has been widely studied regarding its capacity to differentiate between healthy subjects and periodontal patients [350]. To date, there are several chair-side tests (PerioSafe® and ImplantSafe®) whose results have been validated in different countries. These tests measure the levels of MMP8 in saliva (PerioSafe®) or in the sulcular fluid of the implants (ImplantSafe®) to carry out the diagnosis [353]. Although in the literature are many works analysing and comparing different cytokine profiles in healthy and periodontal patients, only a few studies manage to develop predictive models.

Since periodontitis is in itself a persistent inflammation, pro-inflammatory cytokines are the most analysed as possible biomarkers,

with IL1 being again the most studied. Due to the accessible collection of the sample, and the fact that diagnostic tests should facilitate the work of the professionals (e.g. dentists), most of the studies analysing diagnostic accuracy are in saliva.

To the best of our knowledge, there are only two studies evaluating cytokines in GCF from an accuracy point of view. IL1 was studied in both of these studies, with different results. Baeza et al. [303] studied different cytokines in healthy subjects and patients with chronic periodontitis. Both IL1 and IL6 had high levels of discrimination (AUC=0.92, and AUC=0.93, respectively). However, TNFalpha, although being a pro-inflammatory cytokine, did not obtain good results (AUC=0.64).

The other study, conducted by Kitamura et al. [354], also analysed different biomarkers, including IL1apha and IL1beta, in the GCF of periodontal patients to attempt to discriminate between active and inactive sites. The sensitivity of IL1alpha and IL1beta alone was very low, 15% and 23% respectively (both with a specificity of 100%); however, the combination of both together with PGE2, three forms of collagenase and LPS obtained a sensitivity of 62% and specificity of 100%. Disagreements between studies could be due to differences within participants: Baeza et al. [303] included 11 healthy and 13 periodontal patients, reporting their ages and smoking percentage; Kitamura et al. [354], however, included only ten periodontal patients, differentiating between active sites (CAL >2 mm) and inactive sites without bone loss and did not provide descriptive information about them.

On the other hand, we found most of the studies related to diagnostic accuracy and cytokines in saliva samples. As mentioned before, many of them focus on IL1beta or a combination of IL1beta and other markers.

Isaza-Guzmán et al. [355] analysed salivary levels of IL1beta in healthy patients and two types of periodontal patients (aggressive and chronic). IL1beta levels in periodontal patients were elevated

comparing healthy patients, and although they did not analyse sensitivity and specificity, the ORs was significantly elevated in both chronic (6.22) and aggressive patients (4.82). In another study comparing healthy controls and periodontitis patients, the OR for IL1beta in saliva was also significantly higher in chronic periodontitis (OR=5.40) [307].

Afacan et al. [356] studied the higher salivary levels of IL1beta in patients with periodontitis (aggressive and chronic) comparing to controls and obtained the excellent AUC value of 0.95 with 80% of sensitivity and 90% specificity. In the same line of results, Sánchez et al. [357] obtained an AUC value of 0.96 with a sensitivity and specificity of 78% and 100% respectively, for the diagnosis of periodontitis using salivary IL1beta. Besides, in another study, the discrimination of IL1beta in saliva was also very good; in patients with chronic periodontitis, the AUC value was 0.80, while in the case of aggressive periodontitis, the AUC was 0.78 [355].

Ebersole et al. [358] also investigated IL1beta, as well as TNFalpha, IL6 and IFNalpha in saliva from periodontal and healthy patients. IL1beta and IL6 had good sensitivity and specificity (from 0.88 to 0.97), and a positive predictive value (PPV) and an AUC value higher than 0.95. IFNalpha, although showed a fair value of PPV and specificity, had reduced sensitivity (0.54). Like in GCF, TNFalpha did not have good AUC (0.63) and could not discriminate well between groups. In another paper from the same research group, IL1beta and IL6 obtained AUC values of 0.83 and 0.849, respectively. For these cytokines, sensitivity and specificity levels were acceptable (from 0.752 to 0.780), surpassing other biomarkers included such as MMP8. PPV and negative predictive value (NPV) were also obtained, and both in IL1beta and IL6 were higher than 0.7 [272].

In another study with periodontal and healthy patients, the authors combined different biomarkers to obtain the highest level of discrimination with good sensitivity and specificity. IL1beta, together with IL1ra, TNFalpha and MMP9 obtained the highest AUC (0.880)

followed by the combination of IL1beta, IL1ra and MMP9 (AUC = 0.853) [307].

Other research groups also combined different biomarkers or even bacteria to obtain higher levels of discrimination. In a 2009 study, IL1beta alone did not obtain good sensitivity and specificity; however, combining IL1beta, MMP9 and *T. denticola,* the AUC value was 0.9, and the OR was 13.2 [312]. For its part, Ebersole et al. [272] combined IL1beta and IL6 to discriminate between periodontitis and healthy or gingivitis patients; the more significant discrimination with higher sensitivity and specificity were comparing health and periodontitis (accuracy [ACC]=0.79), against periodontitis and gingivitis (ACC=0.76).

So far, although there is a need for more scientific evidence in this regard, data reinforce the hypothesis that cytokines may be accurate biomarkers for periodontitis. Furthermore, the combination of biomarkers seems to improve their ability to diagnose periodontitis. This fact makes them even more interesting as it allows for many combinations to create the most reliable predictive model.

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JUSTIFICATION AND OBJECTIVES

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Justification and Objectives

In periodontics, the diagnosis of periodontitis is a crucial element in the success of treatment, as the progression of the disease causes an irreversible loss of periodontal structures [1]. Traditional clinical measures are the best currently available for diagnosing and monitoring the health-periodontitis states in most patients, probably because they respond favourably to the fundamental principles of periodontal care [2].

Nevertheless, these parameters can only assess the experience and severity of periodontitis, and no reliable information can be obtained regarding the current activity of the disease and its future course [3]. Accordingly, clinical monitoring is time-consuming, subject to considerable measurement error and is often poorly tolerated by patients [4]. As a consequence, researchers are striving to find faster, more sensitive and specific tools based on quantifiable biomarkers in oral fluids, which could supplement or, in some cases, replace the conventional clinical measurements for diagnosis of periodontitis [5,6].

Healthcare professionals looking for evidence about diagnostic tests may turn to systematic reviews of diagnostic test accuracy [7]. Despite being a subject of great interest to the scientific community, there are no systematic reviews and meta-analyses on diagnostic accuracy that reveal which molecules in the oral fluids are most promising biomarkers for the diagnosis of periodontitis.

The complex etiopathogenesis of periodontal diseases has been studied for years in the literature [8,9]. There is a large number of molecules involved during the different disease states, among which we would like to highlight the vital role of inflammatory mediators,

including cytokines [10]. As discussed in the Introduction of this Thesis, numerous papers have reported the measurement of cytokines in gingival crevicular fluid (GCF) and saliva, confirming that there is a distinct cytokine profile for patients with periodontitis [11,12].

However, the existence of a periodontitis-associated biomarker profile in the oral fluids does not indicate its diagnostic capacity. Investigations of diagnostic capacity require the design of a specific accuracy study, which provides estimates of test performance (e.g., sensitivity and specificity) [13]. In this sense, there is scarce evidence in the literature of the development, validation, or updating of oral cytokine-based predictive models for diagnosing periodontitis using appropriate predictive modelling techniques [14]. Besides, the influence of smoking on the diagnostic capacity of cytokines in oral fluids has not yet been evaluated.

Consequently, the lack of evidence on the diagnostic accuracy of cytokines and other molecular biomarkers in oral fluids motivated us to initiate and develop this area of research. In the current Thesis, we have used meta-analysis methodology to systematically review and analyse available data in both GCF and saliva, in order to identify potential biomarkers and evaluate their degree of reliability and potential diagnostic robustness. Next, we have performed experimental work in GCF fluid samples to explore and detect potential biomarkers of disease, developing their corresponding predictive models. Finally, we have used all the above information to select a potential candidate diagnostic component in saliva, testing its discriminatory efficacy in an independent set of patients. Therefore, in this PhD dissertation, we propose the following specific objectives:

1) To analyse, using a meta-analytical approach, the diagnostic accuracy of molecular biomarkers in GCF for the detection of periodontitis in systemically healthy subjects.

2) To analyse, using a meta-analytical approach, the diagnostic capacity of molecular biomarkers in saliva for the detection of periodontitis in systemically healthy subjects.

3) To obtain GCF cytokine-based predictive models that could be used to distinguish systemically healthy subjects with periodontitis from those with periodontal health, developing their corresponding clinical application nomograms and describing their apparent and corrected measures of discrimination and classification.

4) To determine the diagnostic thresholds of periodontitis derived from the best GCF cytokine-based and GCF cytokine ratio-based models in systemically healthy non-smokers and smokers, describing apparent and corrected measures of discrimination and classification.

5) To obtain predictive models based on salivary interleukin 1beta that could be used to distinguish systemically healthy subjects with untreated periodontitis from those with periodontal health and treated periodontitis, differentiating by the smoking status and developing their corresponding clinical application nomograms. The different diagnostic thresholds of periodontitis and performance measures are also described.

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OBJECTIVE 1

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Objective 1. Accuracy of Single Molecular Biomarkers in Gingival Crevicular Fluid for the Diagnosis of Periodontitis in Systemically Healthy Subjects: A Systematic Review and Meta-Analysis

1.1. ABSTRACT

Aim: To analyse, using a meta-analytical approach, the diagnostic accuracy of molecular biomarkers in gingival crevicular fluid (GCF) for the detection of periodontitis in systemically healthy subjects.

Material and Methods: Studies on GCF molecular biomarkers providing a binary classification table (or sensitivity and specificity values and group sample sizes) in individuals with clinically diagnosed periodontitis were considered eligible.

The search was performed using six electronic databases. The methodological quality of studies was assessed through the tool quality assessment of diagnostic studies (QUADAS-2). Meta-analyses were performed using the hierarchical summary receiver operating characteristic (HSROC) modelling, which adjusts classification data using random-effects logistic regression.

Results: The included papers identified 36 potential biomarkers for the detection of periodontitis and for 4 of them meta-analyses were performed. The median sensitivity and specificity were: for MMP8, 76.7% and 92.0%; for elastase, 74.6% and 81.1%; for cathepsin, 72.8% and 67.3% respectively. The worst estimates of sensitivity and specificity were for trypsin (71.3% and 66.1%, respectively).

Conclusions: MMP8 showed good sensitivity and excellent specificity, which resulted in this biomarker being clinically the most useful or effective for the diagnosis of periodontitis in systemically healthy subjects.

Clinical Relevance: Healthcare professionals looking for evidence about diagnostic tests may turn to systematic reviews of diagnostic test accuracy. The findings deriving from the present systematic review/meta-analysis demonstrated MMP8 and elastase were the most researched GCF biomarkers, showing both good capabilities to distinguish periodontitis patients and MMP8 an excellent capability to distinguish non-periodontitis patients. Considering its performance results, MMP8 was clinically the most useful or effective biomarker for diagnosis of periodontitis in systemically healthy subjects.

1.1.1. Keywords

Systematic review; meta-analysis; diagnostic accuracy; molecular biomarkers; gingival crevicular fluid; periodontitis.

1.1.2. Declaration of Conflict of Interest

The doctoral candidate and the rest of the authors of the present study declare that they have no conflict of interest concerning the objectives proposed in this chapter.

1.1.3. Funding

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1.2. INTRODUCTION

1.2.1. Target Condition Being Diagnosed

In 2010, severe periodontitis was estimated to be the sixth most prevalent disease in the world, affecting 743 million people and with an age-standardised incidence of 701 cases per 100,000 person-years [1]. In addition to its high prevalence, periodontitis has become a disease of considerable medical relevance due to the increasing evidence on the bidirectional connection with the pathogenesis of various conditions and systemic diseases of high morbi-mortality such as diabetes, coronary heart disease or metabolic syndrome [2-4].

A new classification of periodontal and peri-implant diseases and conditions has recently been established [5,6]. A 2017 working group agreed that consistent with current knowledge on pathophysiology, three forms of periodontitis can be identified: periodontitis (the forms of the disease previously recognised as “chronic” or “aggressive”, now grouped under a single category); necrotising periodontitis; and periodontitis as a manifestation of systemic disease. This classification framework is characterised by a multidimensional staging and grading system [5,6]. Stages I to IV are defined according to severity, the complexity of the management required and the extent of the disease (localised or generalised). Grades A to C correspond to three categories of progression (slow, moderate and rapid based on direct and indirect evidence). Recognised risk factors (smoking and diabetes) are used as grade modifiers [6].

Periodontitis patients remain so for life, even following successful therapy, and require life-long supportive care to prevent the recurrence of the disease [7]. The condition is usually treated by debridement and other mechanical means that can involve surgery, the removal or reduction of recognised risk factors and appropriate periodontal maintenance. New treatment modalities that are actively explored include antimicrobial therapy, host modulation therapy, laser therapy and tissue engineering for tissue repair and regeneration [8].

1.2.2. Index Test(s)

In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [9].

Gingival crevicular fluid (GCF) is a body fluid that can be easily collected and whose composition is the result of the interplay between the bacterial biofilm and the cells of the periodontium. GCF is the type of sample that most accurately reflects the physiopathological condition of the gingival sulcus and is therefore considered to be the most promising medium for the detection of molecular biomarkers associated with periodontitis [10,11].

An ideal GCF biomarker of periodontitis must be able to: diagnose the presence of the condition; reflect the severity of the disease; predict the progress of the disease; monitor the response to treatment [12]. As a result, and within the field of accuracy, three types of study can be differentiated: 1) diagnostic accuracy studies, which are focused on the analysis of the capability of an index test (in this case, GCF molecular biomarkers) to distinguish patients who have a target condition from those who do not (in this case, periodontitis *versus* periodontal health); 2) prognostic accuracy studies that test information that is used to identify patients who will have an event later on, such as disease progression (the event has not happened at the time the test was taken); 3) predictive accuracy studies, where a test is used to identify patients who will benefit from treatment and those who will not [13]. According to its purpose in the diagnosis of a disease, an index text can be proposed as a triage test, an add-on test, or a replacement for an existing test or test strategy [14].

1.2.3. Clinical Pathway

There is a generalised premise in medicine: the "earlier the disease is diagnosed, more likely it is to be cured successfully". In Periodontics, the first challenge in treating periodontitis is a timely and accurate

diagnosis, as the loss of periodontal bone and soft tissue is incremental and largely irreversible [15].

Traditional clinical measures are informative for evaluating the severity of periodontitis and the response to therapy, and these include: the presence of plaque or the level of oral hygiene; the gingival inflammation and bleeding on probing (BOP); the probing pocket depth (PPD) and suppuration; the clinical attachment loss (CAL); and the radiographic bone loss (BL) [16]. These clinical and radiographic parameters are the best currently available for diagnosing and monitoring the health-disease states in most patients, probably because they respond favourably to the fundamental principles of periodontal care [6].

Nevertheless, these clinical parameters are neither sensitive nor specific enough to identify the current state of disease activity or to predict its future [17]. The BOP parameter is still the best negative predictor of periodontitis activity, with its absence predicting the lack of tissue destruction, although it does produce too many false positives (low sensitivity value) [17]. The evaluation of the CAL measures past episodes of bone destruction and requires a 2- to 3-mm threshold change before a site can be recognised as having developed a significant breakdown [18].

On the other hand, an accurate diagnosis (at specific sites and of the patient overall) requires the recording of all these clinical parameters at six locations per tooth (whether affected or not), which a time-consuming procedure that is dependent on the professional's clinical experience (error-prone measures). Furthermore, this tedious process is often poorly tolerated by patients, as it needs to be repeated regularly on recall visits to monitor the disease course [19].

Accordingly, advances in diagnostic research into periodontal diseases are moving towards the development of reliable, innovative, simple and non-invasive diagnostic methods based on GCF biomarkers for identifying current disease activity, differentiating active from

inactive sites, predicting further disease progression, and monitoring the response to periodontal therapy [11,20].

1.2.4. Rationale

The primary hallmark of periodontitis, namely the destruction of periodontal tissue, is widely accepted to be the result of a chronic inflammatory host immune response caused by a polymicrobial dysbiosis [21,22]. Several papers have reported the measurement of different molecular mediators in GCF, confirming that there is a distinct biomarker profile for patients with different types of periodontal disease [23,24]. Other authors have demonstrated how this profile could be altered after periodontal treatment [23].

The existence of a periodontitis-associated biomarker profile does not in any way indicate its diagnostic capability. Investigations of diagnostic capability require the design of a specific accuracy study, which provides estimates of test performance (e.g., sensitivity and specificity) [25]. Healthcare professionals looking for evidence about diagnostic tests may turn to systematic reviews of diagnostic test accuracy. However, at present, we have not identified any systemic review/meta-analysis of the accuracy of molecular biomarkers in GCF for the diagnosis of periodontitis.

Consequently, this review aims to evaluate the accuracy of single molecular biomarkers detected in GCF for diagnosing periodontitis in systemically healthy subjects.

1.3. MATERIAL AND METHODS

This systematic review/meta-analysis was prepared according to the Cochrane handbook on systematic reviews of diagnostic test accuracy, version 1.0.0 [26], and the PRISMA-DTA statement [25]. The completed PRISMA-DTA checklist is shown in Appendix S1.

The protocol was registered at the International Prospective Register of Systematic Reviews (PROSPERO) under the number CRD42018106045.

1.3.1. PICO Question

The formulated PICO question (patient, index test, comparison, outcome) was as follows: “In systemically healthy subjects, does the expression of single molecular biomarkers in GCF shows diagnostic capability of periodontitis when compared to clinical parameters?”

1.3.2. Criteria for Considering Studies for this Review

1.3.2.1. Types of Accuracy Studies

Studies (whether cross-sectional, longitudinal or interventional) on molecular biomarkers (index tests) in GCF that provided results on diagnostic accuracy in individuals with clinically diagnosed periodontitis (reference standard) were eligible for inclusion. Excluded were studies that did not report: 1) a contingency table for binary classification (2x2 table that includes a number of: true positives, true negatives, false positives and false negatives); or 2) sensitivity and specificity values and sample sizes of the control and target conditions from which the estimation of classification tables was possible. Prognostic and predictive accuracy studies were also excluded.

1.3.2.2. Participants

The participants included in this review were patients of any age without an explicit diagnosis of systemic disease and with a clinical periodontal diagnosis. Studies on patients with clearly defined syndromes or systemic diseases/conditions, as well as those on animal experimentation or *in vitro* models, were excluded.

1.3.2.3. Control and Target Conditions

Following the classification of periodontal diseases and conditions established by Armitage [27], the target conditions evaluated were chronic and aggressive periodontitis, regardless of the extent of the disease and the degree of severity. Studies whose target conditions were gingivitis, peri-implantitis or periodontal conditions other than chronic or aggressive periodontitis were excluded.

For the control condition, patients with a clinical diagnosis of periodontal health and gingivitis were considered. Studies of periodontitis patients, in which the control and target conditions were defined at the site level according to PPD, CAL or BL parameters, were also included.

1.3.2.4. Reference Standard

The reference standard for the diagnosis of a periodontal condition was based on only clinical parameters (PPD or CAL) or clinical and radiographic parameters (BL), irrespective of the diagnostic benchmarks applied. Consequently, in the absence of homogeneous criteria, any definition based on the author's reported criteria was accepted. A clinical periodontal diagnosis was viewed as a binary aspect of two categories, control and target condition, which was established at the patient- or site-level. A patient-level example would be subjects with periodontal health *versus* those with moderate chronic periodontitis. At the site-level, this would be periodontitis patients with periodontal sites with a CAL <2 mm *versus* those same patients with periodontal sites with a CAL ≥2 mm.

Studies that do not detail any reference standard for the diagnosis of the periodontal condition were ineligible for inclusion in this review, as were those that did not assess the periodontal status of the patients using at least one clinical parameter (either PPD or CAL). Analyses of diagnostic classifications at the site-level, whose reference standard was defined exclusively by parameters associated with the gingival inflammation, were also excluded.

1.3.2.5. Index Test(s)

Any single molecular biomarker detected in GCF, which was analysed from an accuracy analysis perspective, was considered to be an index test. Accuracy studies on multi-biomarkers or those detected in other fluids (e.g., blood) were excluded.

1.3.2.6. Other Exclusion Criteria

The following types of study were excluded: thesis, dissertations, reviews, letters, personal opinions, book chapters, short communications, conference abstracts and patents. Other considerations used as exclusion criteria were the following: (i) no restrictions on the publication date of the papers, the setting or the publication status; and (ii) the articles had to be in English.

1.3.3. Search Methods for the Identification and Selection of Studies

1.3.3.1. Information Sources and Search Strategy

The search was conducted through the following electronic databases: PubMed (Medline), Embase, the Cochrane Central Register of Controlled Trials and Trial Protocols, Scopus, Lilacs, and Web of Sciences (WoS). The search strategies were created with input from the authors, following recommendations established by the Cochrane Group for Systematic Reviews of Diagnostic Test Accuracy [26]. As a consequence, the search strategy to identify accuracy studies involved three sets of terms: 1) terms to search for the target condition (periodontitis); 2) terms to identify the index tests (molecular biomarkers) under evaluation; 3) terms to establish the type of oral sample analysed (GCF). To reduce the loss of any relevant studies, any search filter based on methodological terms was avoided. Checks of the references of the included studies and other relevant reviews on the topic were also performed. The search strategy used in the different electronic databases was performed on october 25th, 2018, and is detailed in Table 1.

Table 1. Search strategy applied in the different databases.

• **TERMS FOR TARGET CONDITION**		
1. Periodontitis 2. Periodontal 3. 1 or 2 AND		
• **TERMS FOR THE TYPE OF ORAL SAMPLE ANALYSED**		
4. Fluid AND		
• **TERMS FOR THE INDEX TESTS**		
5. Amino acid	15. Activating factor	54. Hydroxyproline
6. Antibody	16. Adipocytokine	55. Interferon
7. Enzyme	17. Adiponectin	56. Interleukin
8. Immunoglobulin	18. Albumin	57. Keratin
9. Marker	19. Aminopeptidase	58. Lactoferrin
10. Mediator	20. Aminotransferase	59. Laminin
11. Metabolite	21. Amylase	60. Leptin
12. Peptide	22. Antitrypsin	61. Leukotriene
13. Protein	23. Arginase	62. Lysozyme
14. Substance	24. Arylsulfatase	63. Macroglobulin
	25. Ascorbate	64. Melatonin
	26. Calcium	65. Metalloproteinase
	27. Calgranulin	66. Microglobulin
	28. Calprotectin	67. Myeloperoxidase
	29. Cathepsin	68. Neopterin
	30. CD14	69. Neurokinin
	31. Chemokine	70. Nitrate
	32. Chitinase	71. Nitric oxide
	33. Chondroitin	72. Nitrite
	34. Collagenase	73. Osteocalcin
	35. Complement C	74. Osteonectin
	36. Cortisol	75. Osteopontin
	37. Creatine	76. Osteoprotegerin
	38. Creatinine	77. Peptidase
	39. Cystatin	78. Peroxidase
	40. Cytokine	79. Phosphatase
	41. Dehydrogenase	80. Plasminogen

	42. Dipeptidylpeptidase	81. Prostaglandin
	43. Elastase	82. Protease
	44. Esterase	83. Proteinase
	45. Fibronectin	84. Pyridinoline
	46. Gingipain	85. RANKL
	47. Glucuronidase	86. RANTES
	48. Glycosaminoglycan	87. Resistin
	49. Glycosidase	88. Stromelysin
	50. Growth factor	89. TIMP
	51. Hexosaminidase	90. Transferrin
	52. Hyaluronic	91. Urate
	53. Hydroxydeoxyguanosine	92. Visfatin
93. 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22 or 23 or 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32 or 33 or 34 or 35 or 36 or 37 or 38 or 39 or 40 or 41 or 42 or 43 or 44 or 45 or 46 or 47 or 48 or 49 or 50 or 51 or 52 or 53 or 54 or 55 or 56 or 57 or 58 or 59 or 60 or 61 or 62 or 63 or 64 or 65 or 66 or 67 or 68 or 69 or 70 or 71 or 72 or 73 or 74 or 75 or 76 or 77 or 78 or 79 or 80 or 81 or 82 or 83 or 84 or 85 or 86 or 87 or 88 or 89 or 90 or 91 or 92		
94. 3 AND 4 AND 93		

1.3.3.2. Selection of Studies Using a Dual Procedure: Data Mining and Manual Methods

The manipulation of the data identified in the searches was carried out using the R software (version 3.4.3) and packages downloaded from the Comprehensive R Archive Network Team [28].

Applying the search strategy with the resulting combination of terms, a total of 176 searches were performed in each database. Each search was downloaded and stored either in a text (txt) or a comma separated values (csv) file. The downloaded files from Cochrane and WoS were txt files, but those from Pubmed, Embase, Lilacs and Scopus were in the csv format.

The types of data stored and the place where they were recorded were different in the downloaded files from each database. We, therefore, applied an R script and ordered and selected a series of data of interest: authors, title, journal name, digital object identifier (DOI)

and Pubmed identifier (PMID). A total of six csv files were created, one for each database.

The DOI is an international standard used to identify electronic documents and is unique for each article. Many papers do not, however, have this type of identifier, particularly the older ones. The PMID is a unique tool used with articles contained in the Pubmed database. Downloaded WoS, Embase and Scopus files included DOIs, while files from Cochrane and Pubmed had PMIDs; files from Lilacs did not have either of the two identifiers. Duplicate articles were removed from each of the six csv files, and then the unique articles were merged into a single csv file.

The DOI or title of the paper was used in combination with the functions of the RISmed package (version 2.1.7) to obtain the PMID from a manuscript that did not initially provide it [29]. If the PMID identified was unique, then the search was admitted as valid. The PMID data were recorded in a file and duplicates were eliminated. The abstracts of the unique articles were downloaded and recorded using the RISmed package.

In order to ensure research reproducibility [30], the abstracts of all the articles were analysed computationally. On a small number of previously selected diagnostic accuracy articles that met the inclusion criteria, it was confirmed that the automatic data mining process detected 100% of these papers.

Only articles with multiple PMIDs, those with a single PMID that did not provide an abstract or those that did not have a designated PMID were analysed manually. The manual selection was conducted by two independent reviewers (NAB, ARI). A series of positive and negative words were defined, both in their singular and plural forms. Words related to aspects of predictive models and binary classification tests were considered positive; those associated with animal experimentation models were regarded as negative (Table 2). The analyses performed included searches with more than one word and its corresponding acronym if it exists.

Table 2. List of positive and negative words used for the selection of candidate articles.

Positive words	accuracy, sensitivity, specificity, threshold, area under curve, receiver operating, operating characteristic, positive predictive value, negative predictive value, true positive, true negative, false positive, false negative, point of care, chairside test, diagnostic test, prognostic test, logistic regression, canonic correlation, odd ratio, neuronal network, support vector machine, performance measure, predictive model, accurate, prediction, regression, discriminant, cluster, clustering, variance
Negative words	dog, cat, animal, mouse, rat, vitro, monkey, pig, rabbit

The number of positive and negative words found in the abstract of each article was counted. Those with at least one positive word and no negative word were selected as candidates for their full text to be assessed. The analysis of the positive and negative words was carried out using the tm package (version 0.7-5), and NLP package (version 0.1-11) [31,32]. The words "review", "overview", "metaanalysis", "meta analysis" and "meta-analysis" were used to identify systematic reviews and meta-analyses on the topic, then revise the references of each review for selecting candidate articles.

1.3.4. Data collection and Analysis

1.3.4.1. Selection of Studies

Following the screening of titles and abstracts, the studies found by the automated process and those detected by both reviewers were merged in a single database, which included the full texts of the candidate articles.

The analysis of the full texts was carried out by two independent reviewers (NAB and ARI). If the reviewers disagreed, the decision about study eligibility was made by trying to reach a consensus between the two reviewers. Any continued disagreement was resolved by discussion with two different reviewers (IT and CBC). The reasons for excluding studies were recorded. Neither of the review authors were blind to the journal titles or the study authors or institutions.

1.3.5. Data Extraction and Management

Four authors (NAB, ARI, IT and CBC) independently extracted data in duplicate using a standardised data collection form. The first two authors focused on the characteristics of the studies, while the second two concentrated on the accuracy data of biomarkers.

In particular, the following data were recorded from each study: the type of accuracy study (cross-sectional, longitudinal or interventional); the characteristics of the patient groups (country, number, age, smoking); the reference standard (clinical and radiographic parameters registered, calibration, diagnostic criteria); the number and type of control and target conditions at the patient- or site-level; the characteristics of the GCF sample (pooled or individual sample, number and type of gingival sites sampled); the index tests (type of molecular biomarker analysed and the technique used for the detection of biomarkers); the accuracy results of the studies (contingency table based on true positives, true negatives, false positives, false negatives, sensitivity and specificity values, and classification thresholds).

1.3.6. Assessment of Methodological Quality

Two review authors (IT and CBC) independently assessed the quality of the included studies using the critical review checklist of the revised Quality Assessment of Diagnostic Studies (QUADAS-2) [33] (Table 3).

Table 3. QUADAS-2 tool modified according to the characteristics of the included diagnostic accuracy studies [33].

QUADAS-2: METHODOLOGICAL QUALITY OF THE DIAGNOSTIC ACCURACY STUDIES			
Item	**Authors' judgement** (yes, no, or unclear)	**Risk of bias** (high, low, or unclear)	**Applicability concerns** (high, low, or unclear)
DOMAIN 1. Patient Selection			
Was a consecutive or random sample of patients enrolled?			
Was a case–control design avoided?			

Did the study avoid inappropriate exclusions?			
Could the selection of patients have introduced bias?			
Are there concerns that the included patients do not match the review question?			
DOMAIN 2. Index Test			
If a threshold was used, was it prespecified?			
Could the conduct or interpretation of the index test have introduced bias?			
Are there concerns that the index test, its conduct, or its interpretation differ from the review question?			
DOMAIN 3. Reference Standard			
Is the reference standard likely to correctly classify the target condition?			
Were the reference standard results interpreted without knowledge of the results of the index test?			
Could the reference standard, its conduct, or its interpretation have introduced bias?			
Are there concerns that the target condition as defined by the reference standard does not match the review question?			
DOMAIN 4. Flow and Timing			
Was there an appropriate interval between index tests and reference standard?			
Did all patients receive the same reference standard?			
Were all patients or subgingival sites included in the analysis?			
Could the patient flow have introduced bias?			

This checklist evaluates the methodological quality of diagnostic accuracy studies over four key domains: 1) patient selection; 2) index test; 3) reference standard; 4) flow and timing of the participants through the study. A "risk of bias" judgement ("high", "low" or "unclear") was established for each domain. If the answers to all the signalling questions within a domain were judged to be "yes", then the domain was determined to be at low risk of bias. If any signalling

question was judged as “no”, the domain was scored as a high risk of bias.

This first part was followed by a judgement about concerns regarding applicability for the patient selection, index test and reference standard domains. The QUADAS-2 checklist was modified following the recommendations of Whiting et al. [33]. As the biomarkers evaluated in the review were quantified using "objective" methods, the question in domain 2 about blinding the test interpreter to the results of the reference standard was deleted.

1.3.7. Qualitative Analysis

The unit of analysis was each binary classification test (2x2 contingency table) of a biomarker in GCF. According to previously established classifications [20,34], biomarkers were grouped into four types: 1) bacterial and host-derived enzymes and their inhibitors; 2) inflammatory mediators and host-response modifiers; 3) tissue-breakdown products and bone-remodelling molecules; 4) others.

In most studies, the biomarker results were reported on a continuous scale, such as the concentration or level of a quantifiable molecule. Consequently, these results were interpreted as positive or negative based on a numerical measurement that was categorised according to a biomarker classification threshold (pre-stated or not). The diagnostic accuracy of a biomarker was assessed by measures of its capability to detect the presence or absence of a target condition (an event that is present at the moment the index test is taken; e.g., at the patient-level, the presence or absence of periodontitis; at the site-level, the presence or absence of periodontal sites with a CAL ≥2 mm).

The establishment of the contingency table as a unit of analysis means that an article could show more than one table according to different control or target conditions and different techniques used for the quantification of the same biomarker. In those situations in which an article showed several contingency tables of the same biomarker for different classification thresholds and the same control and target

conditions and the same applied technique, that table associated with the highest Youden's index value was selected [35].

The true positive, true negative, false positive and false negative values for each classification of a biomarker from a study were entered into an excel spreadsheet; if these data were not detailed in the article, the authors calculated the 2x2 contingency table considering the sensitivity and specificity values and the sample size of the control and target groups. In relation to the precise sensitivity and specificity values provided in the selected articles, the values of the calculated contingency tables mostly showed decimal values, which had to be rounded. Values equal to zero were not allowed in the tables and were replaced by 0.5; in this way, we avoided infinite values in some measures of the binary classification test, e.g., in the diagnostic odds ratio (DOR) or the positive likelihood ratio (LR+) (Table 4). The two previous premises caused differences in the calculated classification values with respect to those described in the papers, with greater differences in series with small sample sizes.

Table 4. Test indicators; extract from De Luca Canto et al. [46].

TEST INDICATORS	DATA INTERPRETATION	REFERENCES
Sensitivity	>80% excellent, 70-80% good, 60-69% fair, <60% poor	No consensus in this regard exists in the literature
Specificity	>90% excellent, 80-90% good, 70-79% fair, <70% poor	No consensus in this regard exists in the literature
LR	LR+>3 and an LR-<0.3 – acceptable diagnostic test accuracy LR+>10 and LR-<0.1 – excellent diagnostic test accuracy	Brockmann et al. [47]
DOR	The value of a DOR ranges from 0 to infinity, with higher values indicating better discriminatory test performance. A value of 1 means that a test does not discriminate between patients with the disease and those without it. Values lower than 1 point to improper test interpretation (more negative tests among the diseased)	Glas et al. [48]
Youden's index (J index)	Youden's Index values close to 1 indicate high accuracy; a value of zero is equivalent to uninformed guessing and indicates that a test has no diagnostic value	Macaskill et al. [49]

Estimates of accuracy were expressed as sensitivity (SENS) and specificity (SPEC) values, and with 95% confidence intervals, for each classification of a biomarker in GCF (index test). They were then displayed as coupled forest plots. These graphics were created with the mada package (version 0.5.8) [36]. Other performance measures, such as the accuracy (ACC), the positive predictive value (PPV), the negative predictive value (NPV), the LR+, the negative likelihood ratio (LR-), the DOR and the Youden's index, were also determined using the data extracted from each article. Definitions and interpretations are detailed in Table 4.

1.3.8. Quantitative Analysis

A meta-analysis was performed when the number of diagnostic classifications of a biomarker in GCF was reported in at least three articles. Because the biomarkers are continuous tests and the included studies all reported a different threshold for test positivity [37], hierarchical summary receiver operating characteristic (HSROC) modelling was used to conduct the meta-analysis.

HSROC modelling is a multivariate method that jointly evaluates sensitivity and specificity using a within-study binomial data structure while accounting for both within- and between-study heterogeneity. The HSROC model directly estimates HSROC parameters, such as accuracy (αi), threshold (θi) and shape (β), as random effects variables, which enables the direct construction of a HSROC curve [38]. This curve includes summary points of sensitivity and specificity (median values), together with their prediction and confidence region. The prediction region refers to potential sensitivity and specificity values that might be found in a future study by describing the full extent of the uncertainty of the summary points. This region can represent between-study heterogeneity [39]. The confidence region is associated with the summary estimates of sensitivity and specificity jointly in the HSROC space, while it also accounts for their inverse association based on the included studies. This region does not, however, reflect the between-study heterogeneity [40].

The calculation of the HSROC model was performed using the HSROC package (version 2.1.8) [41], whereby estimations are carried out using a Bayesian approach, implemented via a Gibbs sampler [41]. The HSROC package used to calculate meta-analyses implements a model for the joint meta-analysis of sensitivity and specificity of the diagnostic test under evaluation, mathematically taking into account the different sample sizes and the possible imperfections of sensitivity and specificity. This hierarchical model takes into account both variability within the study and variability between studies.

With the aim of trying to provide direct evidence for the usefulness or effectiveness of the GCF biomarkers subjected to meta-analytical analysis [42], we presented the summary accuracy data using natural frequencies based on a hypothetical cohort of 1000 patients for different prevalences of periodontitis [43]; subsequently these frequencies were converted into percentage values. These calculations were made, taking into account summary estimates of sensitivity, specificity, and different prevalences of periodontitis.

1.3.8.1. Investigations of Heterogeneity

One of the most important sources of heterogeneity between diagnostic accuracy studies is known as the "threshold effect". This term defines the correlation observed between sensitivity and specificity by varying the threshold for a positive test result [44]. In the present review, the threshold effect was first evaluated graphically by observing the coupled forest plot. If there was a threshold effect, the sensitivity and specificity changed inversely, showing the coupled forest plot as a V or an inverted-V shape. Second, the threshold effect was statistically evaluated by a linear correlation value between sensitivity and the false-positive rate (1-specificity) using the Spearman correlation coefficient. A threshold effect will be present if the correlation value obtained is 0.6 or greater [45].

Additional analyses to explore other sources of heterogeneity associated with the characteristics of the included studies could not be conducted, as none of the biomarkers achieved a minimum of 10

classifications per possible covariable of interest. Due to the small number of papers included in the meta-analyses, no sensitivity analysis could be carried out.

1.4. RESULTS

1.4.1. Study Selection

In total, 8410 articles were obtained from the six databases. Of these, 87.3% of the abstracts were studied using data-mining techniques and the remaining 12.7% using a manual procedure. After selecting those articles with at least one positive word and no negative words, a total of 120 full-text articles were assessed for eligibility. In addition, seven more articles were detected after studying the references from a list of reviews and full-text papers.

In the eligibility phase, 108 articles were excluded for various reasons (Appendix S2), and 19 articles and 69 contingency tables were selected for the qualitative analysis. After applying the established requirements for the meta-analysis, nine articles and 24 contingency tables were selected for the quantitative analysis. A detailed flow chart is shown in Figure 1.

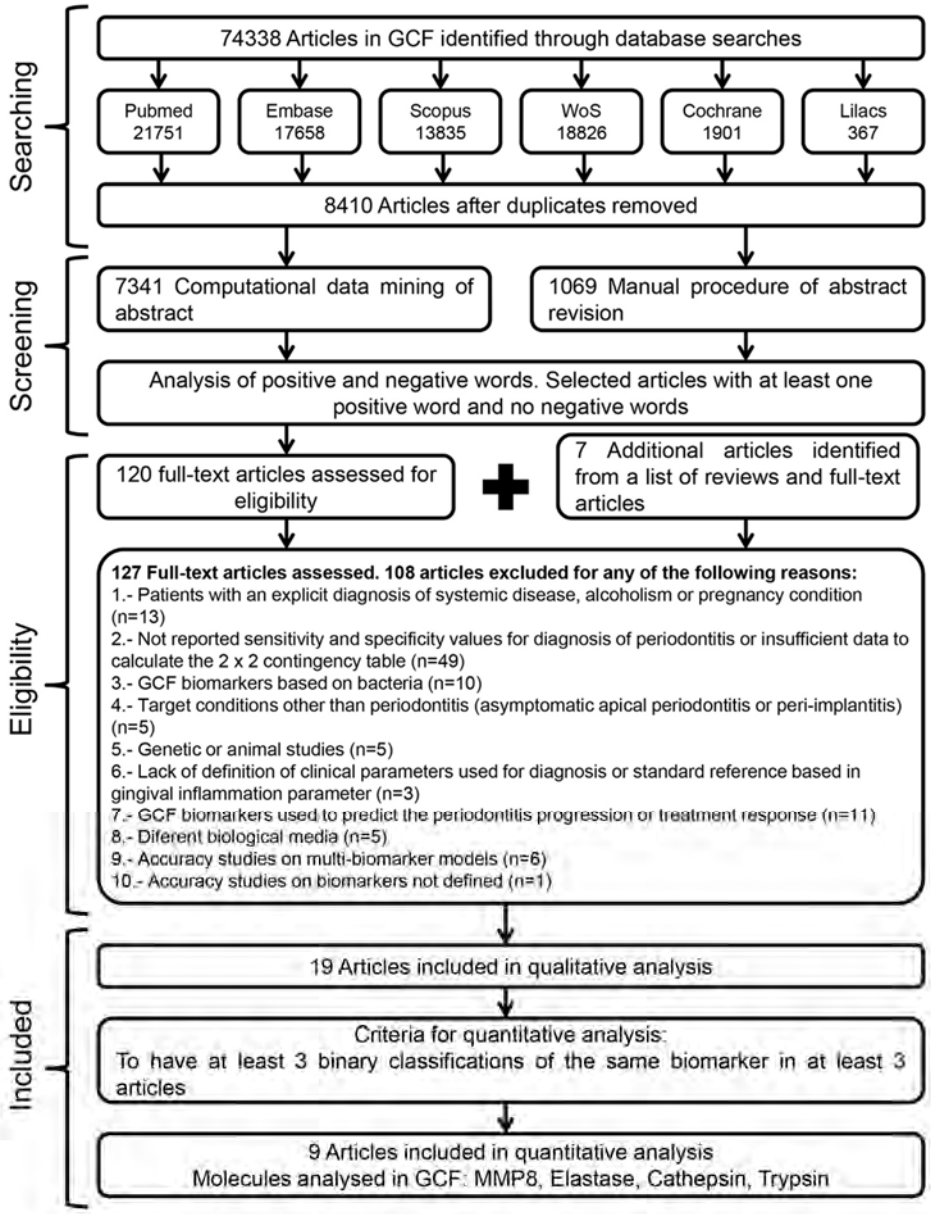


Figure 1. Flow diagram of literature search and selection of the studies adapted from the Cochrane protocol [26].

1.4.2. Characteristics of Diagnostic Accuracy Studies in GCF

Table 5 contains a quantitative summary of the main descriptive characteristics of the included articles. In 8/19 papers (42.1%), the authors investigated the diagnostic accuracy of only one biomarker in GCF, while the remaining 11/19 (57.9%) compared at least two biomarkers. A total of 36 individual molecular biomarkers were identified, of which: 20 (55.6%) were enzymes; eight (22.2%) were inflammatory and host-response mediators; five (13.9%) were periodontal breakdown-related products; three (8.3%) were classified as "others". Twenty-one of the 36 biomarkers (58.3%) were only evaluated in a single article.

Regarding the type of control condition, 59.3% of the classifications corresponded to healthy patients, and 25.0% to the combination of healthy patients and gingivitis patients. When the control condition was defined by subject and subgingival site, 37.8% of the classifications corresponded to subjects with chronic periodontitis and subgingival sites with no CAL. Regarding the type of target condition, 68.7% of the classifications corresponded to patients with chronic periodontitis; when the target condition was defined by subject and subgingival site, 37.8% of the classifications corresponded to subjects with chronic periodontitis and subgingival sites with CAL.

Although in 11/19 papers (57.9%) nothing was specified about the smoking habit of the participants, in 5/19 (26.3%) study groups were composed of both smokers and non-smokers with a predominance of the last-mentioned.

Regarding the GCF collection protocol, the use of paper points and periopapers strips are the most commonly used fluid collection methods (in 37% and 21% of the series, respectively); other aspects such as the storage temperature of the samples, the most frequent is storage at -80ºC for further processing (in 31.6% of the series). The most frequently applied techniques for the detection/quantification of biomarkers were colourimetric or fluorimetric methods (27.0%), multiparametric cytometry (14.9%) or ELISA (10.8%).

Table 5. Biomarkers in GCF analysed, characteristics of controls and target conditions and technique applied for the detection of biomarkers; definition of the index tests included in the meta-analysis.

	Index Test	Number of Classifications /Studies	Control Condition			Target Condition			Technique	Meta-Analysis
			Range of Number		Types	Range of Number		Types		
No	Name (Type)		Patients	Samples		Patients	Samples		Name	
1	MMP8 (E)	13/6	≤30 (12/5) 71-120 (1/1)	≤30 (2/2) 31-70 (7/3) 71-120 (3/3) 121-200 (1/1)	H (4/2) G (3/2) H and G (3/2) CP_H (2/2) C (1/1)	≤30 (12/5) 71-120 (1/1)	≤30 (2/1) 31-70 (3/2) 71-120 (6/1) 121-200 (2/2)	CP (8/2) CP-M-S (3/2) CP_P (2/2)	ELISA (3/3) IFMA (5/3) IC test stick (3/1) Colour/Fluor assay (1/1) NSp (1/1)	Y
2	Elastase (E)	5/5	≤30 (3/3) 71-120 (1/1) 121-200 (1/1)	≤30 (1/1) 71-120 (1/1) ≥201 (3/3)	CP_H (3/3) CP_Non-BL (1/1) CP_Non-PPD (1/1)	≤30 (3/3) 71-120 (1/1) 121-200 (1/1)	31-70 (1/1) 71-120 (1/1) 121-200 (1/1) ≥201 (2/2)	CP_P (3/3) CP_BL (1/1) CP_PPD (1/1)	Colour/Fluor assay (3/3) Elastase substrate assay (1/1) NSp (1/1)	Y
3	Cathepsin (E)	3/3	≤30 (3/3)	≤30 (1/1) ≥201 (2/2)	CP_H (1/1) CP_Non-BL (1/1) CP_Non-PPD (1/1)	≤30 (3/3)	31-70 (1/1) ≥201 (2/2)	CP_P (1/1) CP_BL (1/1) CP_PPD (1/1)	Colour/Fluor assay (3/3)	Y
4	Trypsin (E)	3/3	≤30 (3/3)	≤30 (1/1) ≥201 (2/2)	CP_H (1/1) CP_Non-BL (1/1) CP_Non-PPD (1/1)	≤30 (3/3)	31-70 (1/1) ≥201 (2/2)	CP_P (1/1) CP_BL (1/1) CP_PPD (1/1)	Colour/Fluor assay (3/3)	Y
5	COL (E)	4/2	≤30 (4/2)	≤30 (3/1) 31-70 (1/1)	CP_Non-CAL (4/2)	≤30 (4/2)	≤30 (4/2)	CP_CAL (4/2)	Modified Lamster method (1/1) NSp (3/1)	N
6	Tryptase (E)	2/2	≤30 (2/2)	≥201 (2/2)	CP_Non-BL (1/1) CP_Non-PPD (1/1)	≤30 (2/2)	≥201 (2/2)	CP_BL (1/1) CP_PPD (1/1)	Colour/Fluor assay (2/2)	N

7	DPP (E)	2/2	≤30 (2/2)	≥201 (2/2)	CP_Non-BL (1/1) CP_Non-PPD (1/1)	≤30 (2/2)	≥201 (2/2)	CP_BL (1/1) CP_PPD (1/1)	Colour/Fluor assay (2/2)	N
8	ALP (E)	2/2	≤30 (2/2)	31-70 (2/2)	CP_Non-CAL (1/1) CP-M-S_Non-CAL (1/1)	≤30 (2/2)	≤30 (1/1) 31-70 (1/1)	CP_CAL (1/1) CP-M-S_CAL (1/1)	Spectrophotometry (1/1) Phosphorylation assay (1/1)	N
9	AST (E)	2/2	≤30 (1/1) 121-200 (1/1)	121-200 (1/1) ≥201 (1/1) P	CP-M-S_Non-CAL (1/1) CP_H (1/1)	≤30 (1/1) 121-200 (1/1)	≤30 (1/1) 71-120 (1/1)	CP-M-S_CAL (1/1) CP_P (1/1)	NSp (1/1) AST activity kit (1/1)	N
10	BG (E)	2/2	≤30 (1/1) 31-70 (1/1)	31-70 (2/2)	CP_Non-CAL (2/2)	≤30 (2/2)	≤30 (2/2)	CP_CAL (2/2)	Spectrophotometry (1/1) NSp (1/1)	N
11	MPO (E)	2/2	≤30 (2/2)	≤30 (1/1) 31-70 (1/1)	H (1/1) G (1/1)	≤30 (2/2)	≤30 (1/1) 31-70 (1/1)	CP (1/1) CP-M-S (1/1)	ELISA (2/2)	N
12	Sialidase (E)	2/2	≤30 (1/1) 71-120 (1/1)	≤30 (1/1) 71-120 (1/1)	CP_H (2/2)	≤30 (1/1) 71-120 (1/1)	31-70 (1/1) 121-200 (1/1)	CP_P (2/2)	Colour/Fluor assay (1/1) NSp (1/1)	N
13	IL1alpha (I)	2/2	≤30 (1/1) 71-120 (1/1)	≤30 (1/1) 71-120 (1/1)	H (1/1) CP_Non-CAL (1/1)	≤30 (1/1) 71-120 (1/1)	≤30 (1/1) 71-120 (1/1)	CP-Ge-M-S (1/1) CP_CAL (1/1)	Multiplex cytometry (1/1) NSp (1/1)	N
14	IL1beta (I)	2/2	≤30 (1/1) 71-120 (1/1)	≤30 (1/1) 71-120 (1/1)	H (1/1) CP_Non-CAL (1/1)	≤30 (1/1) 71-120 (1/1)	≤30 (1/1) 71-120 (1/1)	CP-Ge-M-S (1/1) CP_CAL (1/1)	Multiplex cytometry (1/1) NSp (1/1)	N
15	PGE2 (I)	2/2	≤30 (1/1) 31-70 (1/1)	≤30 (2/2)	CP_Non-CAL (2/2)	≤30 (1/1) 31-70 (1/1)	≤30 (2/2)	CP_CAL (2/2)	Radioinmmunoassay (1/1) NSp (1/1)	N
16	Protease (E)	1/1	≤30 (1/1)	31-70 (1/1)	CP-M-S_H (1/1)	≤30 (1/1)	71-120 (1/1)	CP-M-S_P (1/1)	Periocheck test (1/1)	N
17	ProMMP2 (E)	1/1	≤30 (1/1)	31-70 (1/1)	H (1/1)	≤30 (1/1)	31-70 (1/1)	CP (1/1)	Zymo-densitometry (1/1)	N
18	ProMMP9 (E)	1/1	≤30 (1/1)	31-70 (1/1)	H (1/1)	≤30 (1/1)	31-70 (1/1)	CP (1/1)	Zymo-densitometry (1/1)	N

19	MMP9 (E)	1/1	≤30 (1/1)	31-70 (1/1)	H (1/1)	≤30 (1/1)	31-70 (1/1)	CP (1/1)	Zymo-densitometry (1/1)	N
20	TRAP5 (E)	1/1	≤30 (1/1)	31-70 (1/1)	H (1/1)	≤30 (1/1)	31-70 (1/1)	CP (1/1)	Multiplex cytometry (1/1)	N
21	MMP13 (E)	1/1	≤30 (1/1)	31-70 (1/1)	H and G (1/1)	≤30 (1/1)	≤30 (1/1)	CP-M-S (1/1)	Fluorokine E assay (1/1)	N
22	MMP14 (E)	1/1	≤30 (1/1)	31-70 (1/1)	H and G (1/1)	≤30 (1/1)	≤30 (1/1)	CP-M-S (1/1)	Biotrak assay (1/1)	N
23	To-Protein (E)	1/1	121-200 (1/1)	≥201 (1/1)	CP_H (1/1)	121-200 (1/1)	71-120 (1/1)	CP_P (1/1)	BCA protein assay (1/1)	N
24	IL1 (I)	1/1	≤30 (1/1)	31-70 (1/1)	H (1/1)	≤30 (1/1)	31-70 (1/1)	CP (1/1)	Multiplex cytometry (1/1)	N
25	IL6 (I)	1/1	≤30 (1/1)	31-70 (1/1)	H (1/1)	≤30 (1/1)	31-70 (1/1)	CP (1/1)	Multiplex cytometry (1/1)	N
26	TNFalpha (I)	1/1	≤30 (1/1)	31-70 (1/1)	H (1/1)	≤30 (1/1)	31-70 (1/1)	CP-Ge-M-S (1/1)	Multiplex cytometry (1/1)	N
27	IL17A (I)	1/1	71-120 (1/1)	71-120 (1/1)	H (1/1)	71-120 (1/1)	71-120 (1/1)	CP-Ge-M-S (1/1)	Multiplex cytometry (1/1)	N
28	Azurocidin (I)	1/1	≤30 (1/1)	31-70 (1/1)	H and G (1/1)	≤30 (1/1)	≤30 (1/1)	CP-M-S (1/1)	ELISA (1/1)	N
29	Calprotectin (T)	1/1	31-70 (1/1)	71-120 (1/1)	P_H (1/1)	31-70 (1/1)	71-120 (1/1)	P_P (1/1)	IC chip (1/1)	N
30	DKK1 (T)	1/1	≤30 (1/1)	31-70 (1/1)	H (1/1)	≤30 (1/1)	31-70 (1/1)	CP (1/1)	Multiplex cytometry (1/1)	N
31	ON (T)	1/1	≤30 (1/1)	31-70 (1/1)	H (1/1)	≤30 (1/1)	31-70 (1/1)	CP (1/1)	Multiplex cytometry (1/1)	N
32	PTN (T)	1/1	≤30 (1/1)	31-70 (1/1)	H (1/1)	≤30 (1/1)	31-70 (1/1)	CP (1/1)	Multiplex cytometry (1/1)	N
33	OPG (T)	1/1	≤30 (1/1)	31-70 (1/1)	H (1/1)	≤30 (1/1)	31-70 (1/1)	CP (1/1)	Multiplex cytometry (1/1)	N
34	Nitrate (O)	1/1	≤30 (1/1)	≤30 (1/1)	H and G (1/1)	≤30 (1/1)	≤30 (1/1)	P (1/1)	ELISA (1/1)	N
35	Nitric oxide (O)	1/1	≤30 (1/1)	≤30 (1/1)	H and G (1/1)	≤30 (1/1)	≤30 (1/1)	P (1/1)	ELISA (1/1)	N
36	LPS (O)	1/1	≤30 (1/1)	≤30 (1/1)	CP_Non-CAL (1/1)	≤30 (1/1)	≤30 (1/1)	CP_CAL (1/1)	NSp (1/1)	N

Regarding type of control and target conditions: if there is only one abbreviation, it indicates the type of patient; if there are two abbreviations separated by an underscore, it indicates the type of patient and the type of subgingival site, respectively.

1.4.3. Quality Assessment of Diagnostic Accuracy Studies in GCF

Fourteen papers (73.7%) were case-control studies which conditioned patient selection domain was classified as high risk of bias in 16/19 articles (84.2%). However, practically all the studies met the requirements of a “single-gate design”. In the index text domain, the question about the application of a pre-specified threshold was judged as "no" in 15/19 articles (78.9%), indicating a high risk of bias.

The reference standard correctly classified the periodontal condition without knowledge of the results of the index text in 16/19 articles (84.2%) so in these studies, the standard reference domain was associated with a low risk of bias. However, it is important to note that in only six articles (31.6%), the authors mentioned the practice of calibration methods or the participation of calibrated professionals for the recording of clinical parameters. All patients in all included articles received the same reference standard, with 68.4% of them (13/19 articles) having an appropriate interval between the reference standard and the index test; this domain was considered low risk in 11/19 articles (57.9%). Concerns regarding applicability were judged as “low” in all domains of all the articles (Figure 2).

Considering the sample size as an indicator of quality, 83.3% of the contingency tables had ≤30 subjects in each group (control and target condition), while only 13.0% had >70 subjects. At the site-level, larger sample sizes were detected; it was in 27.5% of the contingency tables that ≤30 fluid samples were analysed, while >70 were evaluated in 37.6% of the cases (Table 5).

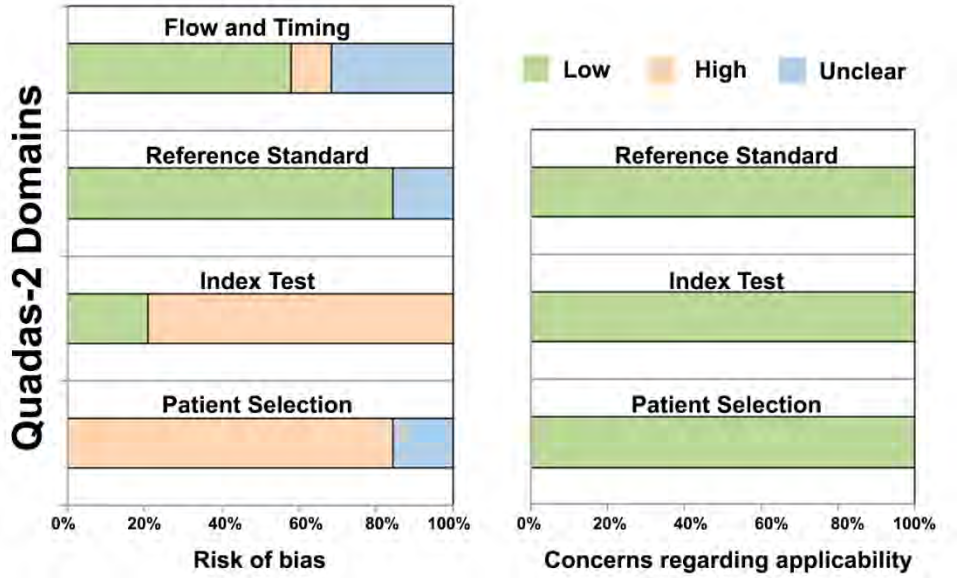


Figure 2. Quality assessment, according to the QUADAS-2 tool: risk of bias and applicability concerns.

1.4.4. Synthesis of the Qualitative and Quantitative Analysis of Four Biomarkers

Only four of the 36 biomarkers had at least three classifications in at least three articles, and it was on these that the meta-analyses were performed. These molecules were all enzymes: matrix metalloproteinase (MMP) 8, 1907 control and target conditions/13 classifications/six articles [50-55]; elastase, 1660/five/five [52,53,56-58]; cathepsin, 1146/three/three [52,56,57]; trypsin, 1146/three/three [52,56,57].

With the exception of trypsin, none of these biomarkers showed a threshold effect, either graphically or statistically. The ACC range (sensitivity and specificity ranges) were: 94.8-72.3% (96.8-64.4% and 98.3-77.6%) for MMP8; 88.9-69.7% (88.3-52.6% and 90.0-61.1%) for elastase; 82.2-53.3% (84.1-55.0% and 79.8-50.0%) for cathepsin; and 72.2-68.0% (80.0-71.0% and 68.1-56.7%) for trypsin (Figures 3A, 3B and Table 6).

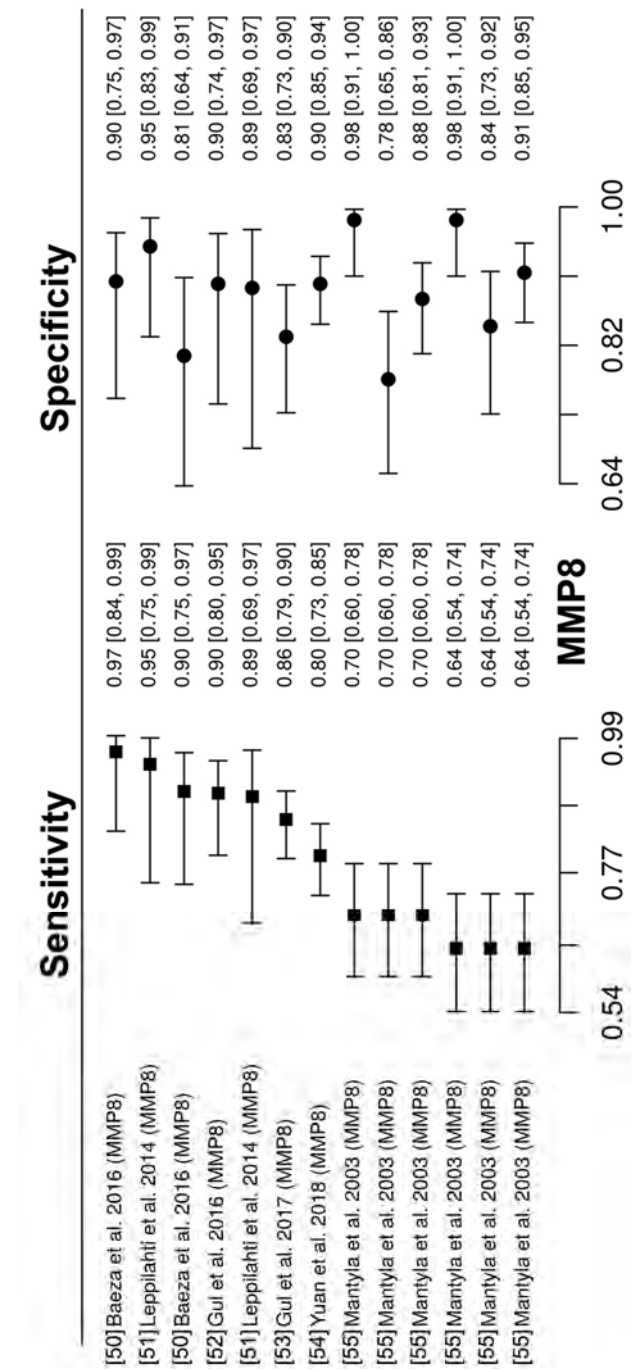


Figure 3A. Coupled forest plot with diagnostic test accuracy (sensitivity, specificity, and 95% confidence interval) of the MMP8 classifications included in the meta-analysis. In the coupled forest plot, sensitivity and specificity values are rounded.

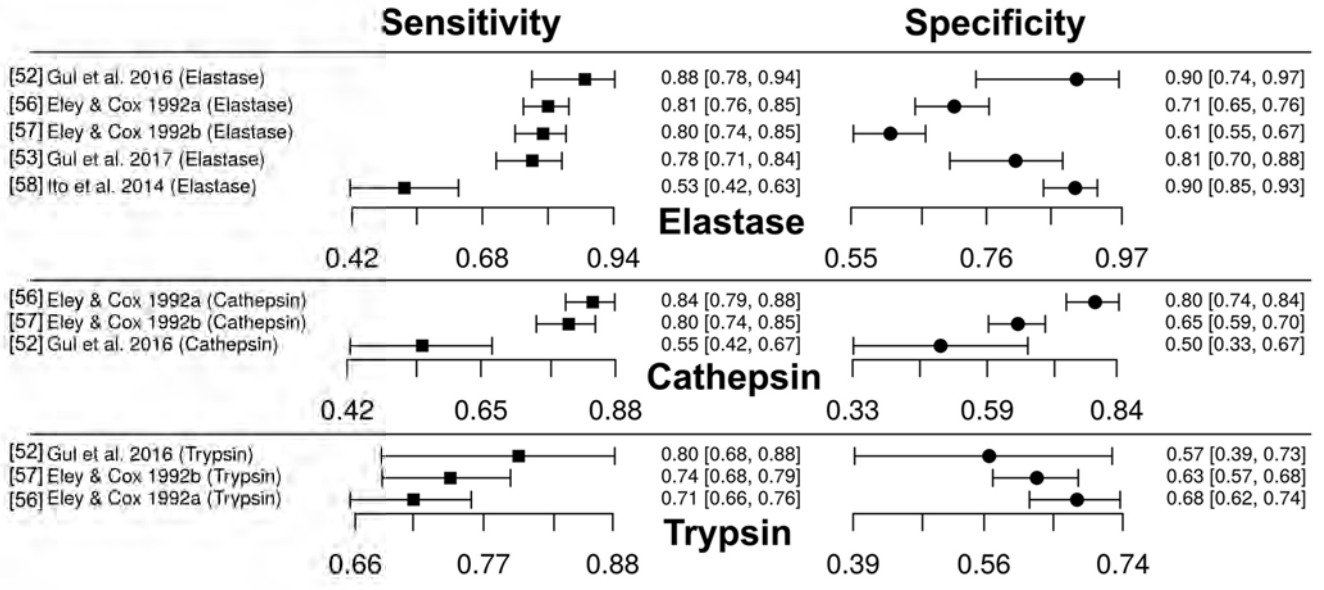


Figure 3B. Coupled forest plot with diagnostic test accuracy (sensitivity, specificity, and 95% confidence interval) of the elastase, cathepsin and trypsin classifications included in the meta-analysis. In the coupled forest plot, sensitivity and specificity values are rounded.

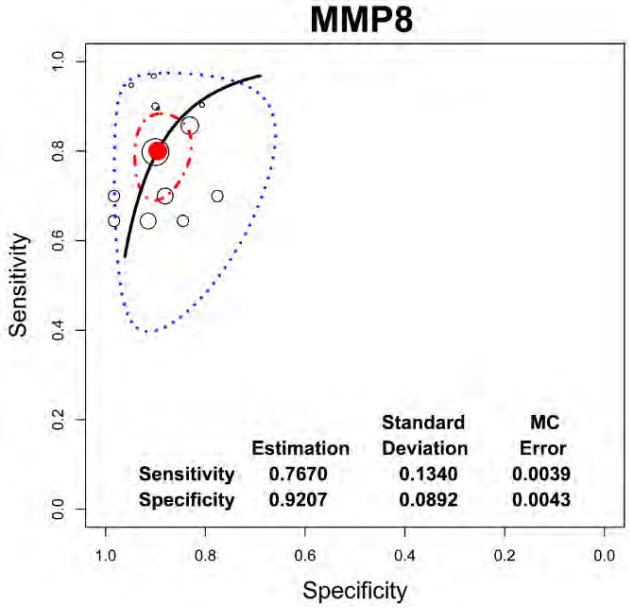
Table 6. Diagnostic test accuracy measurements for each GCF biomarker classification included in the meta-analysis.

Study (first author)	Index Test	No. CC/ No. TC	Threshold (Units)	AUC	ACC (%)	SENS/ SPEC (%)	PPV/ NPV (%)	LR+/ LR-	DOR	J Index
Baeza [50]	MMP8	31/ 31	17.1 (ng/ml)	0.980	93.5	96.8/ 90.3	90.9/ 96.6	10.0/ 0.0	280.0	0.87
Leppilahti [51]		39/ 19	754.1 (ng/ml)	0.970	94.8	94.7/ 94.9	90.0/ 97.4	18.5/ 0.1	333.0	0.90
Baeza [50]		31/ 31	71.0 (ng/ml)	0.920	85.5	90.3/ 80.6	82.4/ 89.3	4.7/ 0.1	38.9	0.71
Gul [52]		30/ 60	104.0 (NSp)	0.910	90.0	90.0/ 90.0	94.7/ 81.8	9.0/ 0.1	81.0	0.80
Leppilahti [51]		19/ 19	135.9 (ng/ml)	0.900	89.5	89.5/ 89.5	89.5/ 89.5	8.5/ 0.1	72.3	0.79
Gul [53]		77/ 154	94.0 (ng/ul)	0.920	84.8	85.7/ 83.1	91.0/ 74.4	5.1/ 0.2	29.5	0.69
Yuan [54]		180/ 178	6.6 (ng/ml)	NSp	84.9	79.8/ 90.0	88.8/ 81.8	8.0/ 0.2	35.5	0.70
Mäntylä [55]		59/ 90	1.0 (mg/l)	NSp	81.2	70.0/ 98.3	98.4/ 68.2	41.3/ 0.3	135.3	0.68
Mäntylä [55]		58/ 90	1.0 (mg/l)	NSp	73.0	70.0/ 77.6	82.9/ 62.5	3.1/ 0.4	8.1	0.48
Mäntylä [55]		117/ 90	1.0 (mg/l)	NSp	80.2	70.0/ 88.0	81.8/ 79.2	5.9/ 0.3	17.2	0.58
Mäntylä [55]		59/ 90	1.0 (mg/l)	NSp	77.9	64.4/ 98.3	98.3/ 64.4	38.0/ 0.4	105.1	0.63
Mäntylä [55]		58/ 90	1.0 (mg/l)	NSp	72.3	64.4/ 84.5	86.6/ 60.5	4.2/ 0.4	9.9	0.49
Mäntylä [55]		117/ 90	1.0 (mg/l)	NSp	79.7	64.4/ 91.5	85.3/ 77.0	7.5/ 0.4	19.4	0.56

Gul [52]	Elastase	30/60	24.0 (NSp)	0.880	88.9	88.3/90.0	94.6/79.4	8.8/0.1	68.1	0.78
Eley [56]		238/290	40.0 (µU/60 s)	NSp	76.5	81.0/71.0	77.3/75.4	2.8/0.3	10.5	0.52
Eley [57]		288/240	40.0 (µU/60 s)	NSp	69.7	80.0/61.1	63.2/78.6	2.1/0.3	6.3	0.41
Gul [53]		77/154	33.0 (ng/ul)	0.870	78.8	77.9/80.5	88.9/64.6	4.0/0.3	14.6	0.58
Ito [58]		205/78	199.6 (µU)	0.762	79.5	52.6/89.8	66.1/83.3	5.1/0.5	9.7	0.42
Eley [56]	Cathepsin	238/290	25.0 (µU/60 s)	NSp	82.2	84.1/79.8	83.6/80.5	4.2/0.2	21.0	0.64
Eley [57]		288/240	25.0 (µU/60 s)	NSp	71.8	80.0/64.9	65.5/79.6	2.3/0.3	7.4	0.45
Gul [52]		30/60	0.8 (NSp)	0.630	53.3	55.0/50.0	68.8/35.7	1.1/0.9	1.2	0.05
Gul [52]	Trypsin	30/60	12.0 (NSp)	0.720	72.2	80.0/56.7	78.7/58.6	1.8/0.4	5.2	0.37
Eley [57]		288/240	3.0 (µU/60 s)	NSp	68.0	74.2/62.8	62.5/74.5	2.0/0.4	4.9	0.37
Eley [56]		238/290	3.0 (µU/60 s)	NSp	69.7	71.0/68.1	73.1/65.9	2.2/0.4	5.2	0.39

Biomarkers are listed according to the sensitivity value (from highest to lowest value).

Figures 4A and 4B show the meta-analyses performed on the four biomarkers in GCF using HSROC modelling. In meta-analyses, the median estimators of sensitivity and specificity ± standard deviation obtained were: 76.7 ± 13.4% and 92.0 ± 8.9% for MMP8; 74.6 ± 20.8% and 81.1 ± 19.2% for elastase; and 72.8 ± 23.5% and 67.3 ± 24.2% for cathepsin. The worst estimated sensitivity and specificity values were for trypsin (71.3 ± 17.5% and 66.1 ± 18.0%). Graphically, MMP8 was the biomarker that presented the narrowest prediction region, in which potential sensitivity and specificity values could be found in a future study. All four biomarkers showed a Markov chain (MC) error value of sensitivity and specificity parameters smaller than 10% of its respective standard deviation, which means a high precision in the estimation of the parameters (Figures 4A, 4B and Appendix S3). Applying the premise of having at least three contingency tables of at least three articles, we performed various meta-analytical analyses of the MMP8 biomarker according to various selection criteria, the results of which are presented in Appendix S4.



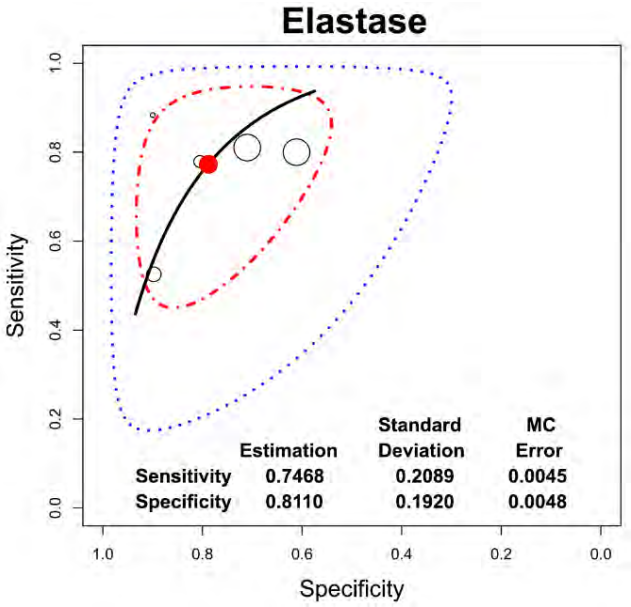
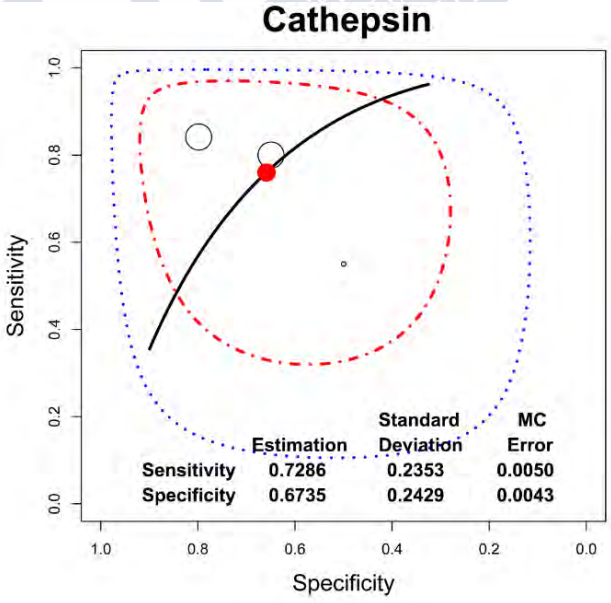


Figure 4A. Meta-analyses performed on MMP8 and elastase in GCF using HSROC modelling.



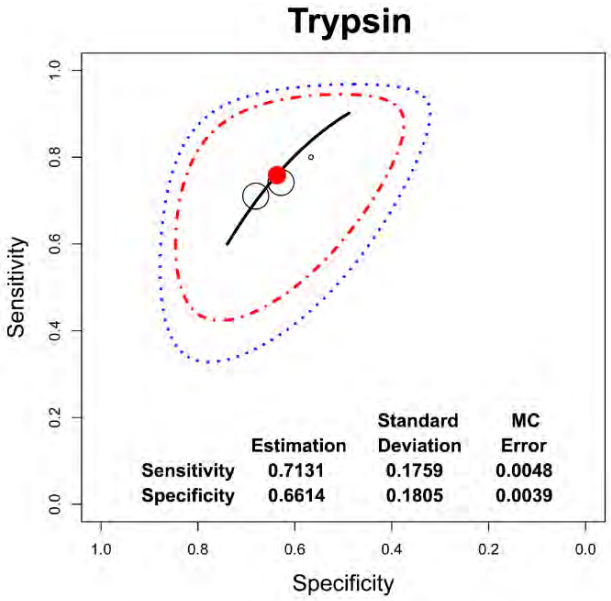


Figure 4B. Meta-analyses performed on cathepsin and trypsin in GCF using HSROC modelling.

HSROC includes summary points of sensitivity and specificity (median values; red-filled point), together with their prediction and confidence region. The prediction region (red dotted area) refers to potential sensitivity and specificity values that might be found in a future study by describing the full extent of the uncertainty of the summary points. This region can represent between-study heterogeneity [39]. The confidence region (blue dotted area) is associated with the summary estimates of sensitivity and specificity jointly in the HSROC space, while it also accounts for their inverse association based on the included studies. This region does not, however, reflect the between-study heterogeneity [40]. Each black circle represents a classification of a biomarker included in the meta-analysis, and its size is proportional to the sample size.

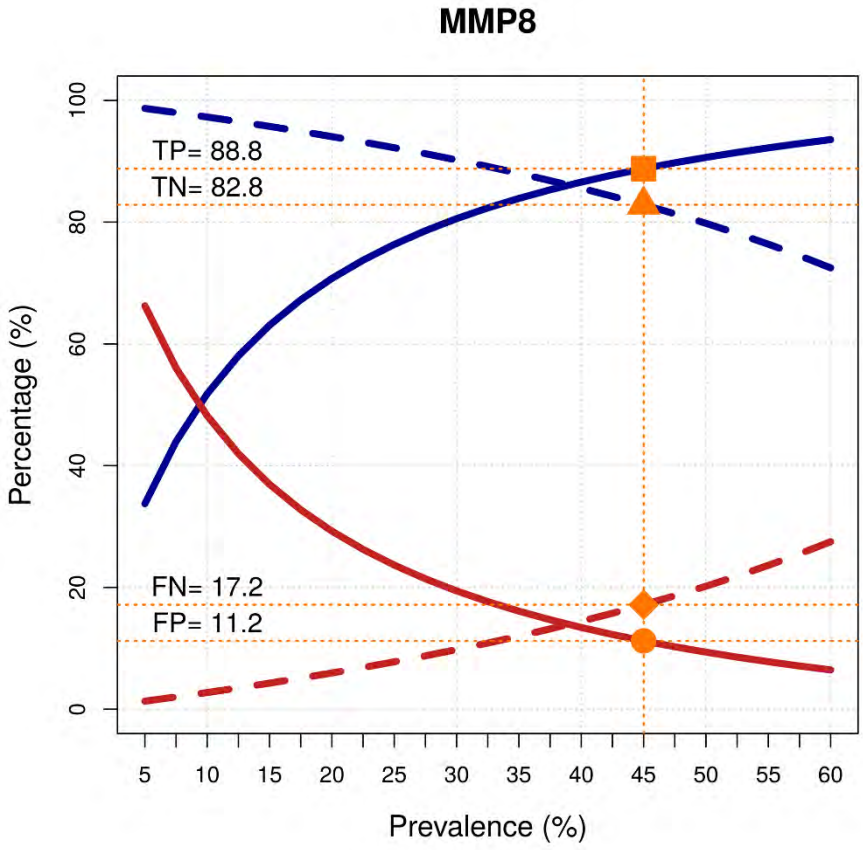


Figure 5A. Expression of summary accuracy data derived from meta-analyses using natural frequencies based on a hypothetical cohort of 1000 patients along different periodontitis prevalence values for the two most studied biomarkers in GCF (MMP8).

The blue line indicates the true cases for the different prevalence of periodontitis (the dashed line, the true negatives; the continuous line, the true positives). The red line indicates false cases for the different prevalence of periodontitis (the dashed line, false negatives; the continuous line, false positives). TP: true positive, test is positive (indicates periodontitis and patient has periodontitis); FP: false positive, test is positive (indicates periodontitis but patient does not have periodontitis); TN: true negative, test is negative (indicates periodontitis not present and patient does not have periodontitis); FN: false negative, test is negative (indicates periodontitis not present but patient has periodontitis).

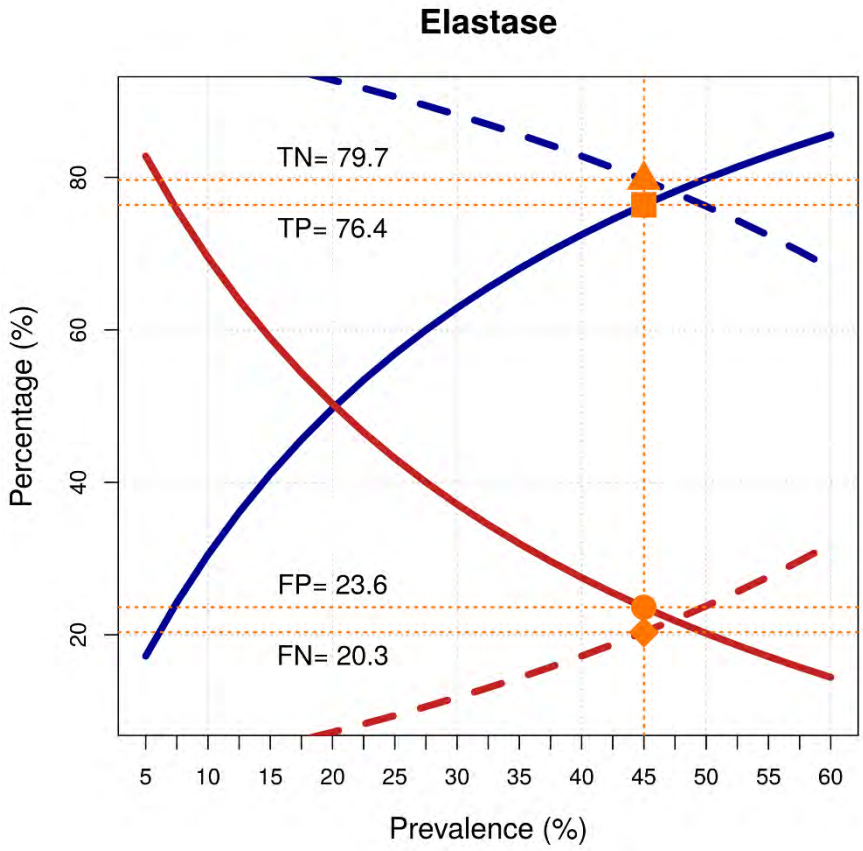


Figure 5B. Expression of summary accuracy data derived from meta-analyses using natural frequencies based on a hypothetical cohort of 1000 patients along different periodontitis prevalence values for the two most studied biomarkers in GCF (elastase).

The blue line indicates the true cases for the different prevalence of periodontitis (the dashed line, the true negatives; the continuous line, the true positives). The red line indicates false cases for the different prevalence of periodontitis (the dashed line, false negatives; the continuous line, false positives). TP: true positive, test is positive (indicates periodontitis and patient has periodontitis); FP: false positive, test is positive (indicates periodontitis but patient does not have periodontitis); TN: true negative, test is negative (indicates periodontitis not present and patient does not have periodontitis); FN: false negative, test is negative (indicates periodontitis not present but patient has periodontitis).

In terms of usefulness or effectiveness of the two most studied GCF biomarkers (MMP8 and elastase), considering a 45% prevalence of periodontitis [59,60], 88.8% of the total MMP8 positive tests would indicate a true positive; while of the total MMP8 negative tests, 82.8% would show a true negative. For an elastase test, these percentages would be 79.7% and 76.4%, respectively (Figures 5A and 5B).

1.4.5. Synthesis of the Qualitative Analysis of the Remaining Biomarkers

If we focus on those diagnostic classifications that presented better ACC values, above 90%, for diagnosis of periodontitis (sensitivity/specificity values), Baeza et al. [50] obtained an ACC of 95.2% (93.5%/96.8%) for ProMMP2 and a value of 95.2% (96.8%/93.5%) for ProMMP9. For other enzymes, Leppilahti et al. [51] detected an ACC of 94.7% (94.7%/94.7%) and 91.4% (94.7%/89.7%) for myeloperoxidase (MPO) and MMP14, respectively. Regarding inflammatory biomarkers, Tomás et al. [72] observed that interleukin (IL)1beta presented an ACC of 93.9% (93.2%/94.6%), while IL1alpha, an ACC of 93.2% (94.5%/91.9%) (Figures 6A, 6B, 6C, 6D and Table 7).

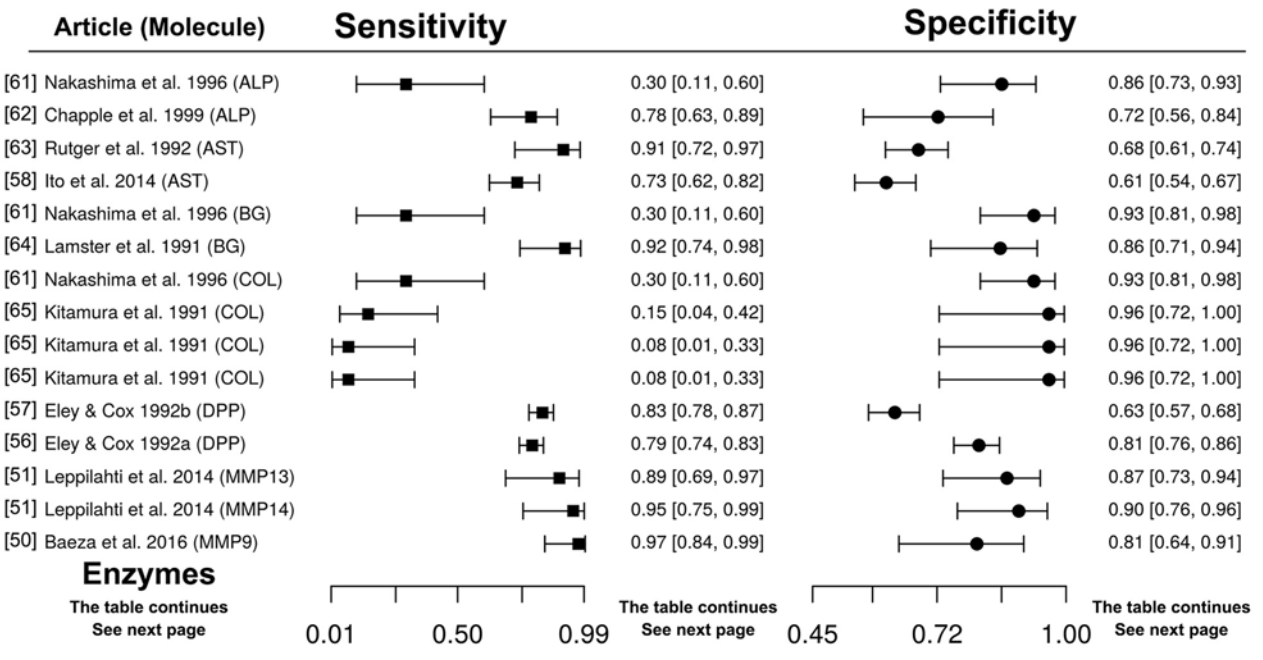


Figure 6A. Coupled forest plot with diagnostic test accuracy (sensitivity, specificity, and 95% confidence interval) of the classifications of the enzymes included only in the qualitative analysis. The biomarkers included in the qualitative analysis were those that presented less than three classifications in less than three articles. In the coupled forest plot, sensitivity and specificity values are rounded.

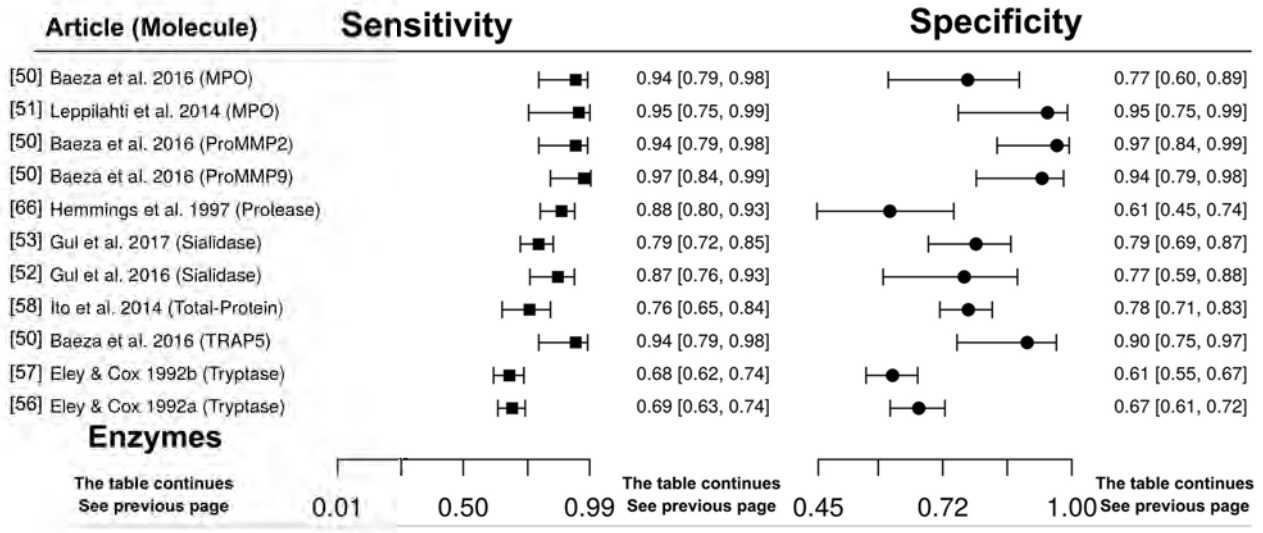


Figure 6B. Coupled forest plot with diagnostic test accuracy (sensitivity, specificity, and 95% confidence interval) of the classification of the enzymes included only in the qualitative analysis.The biomarkers included in the qualitative analysis were those that presented less than three classifications in less than three articles. In the coupled forest plot, sensitivity and specificity values are rounded.

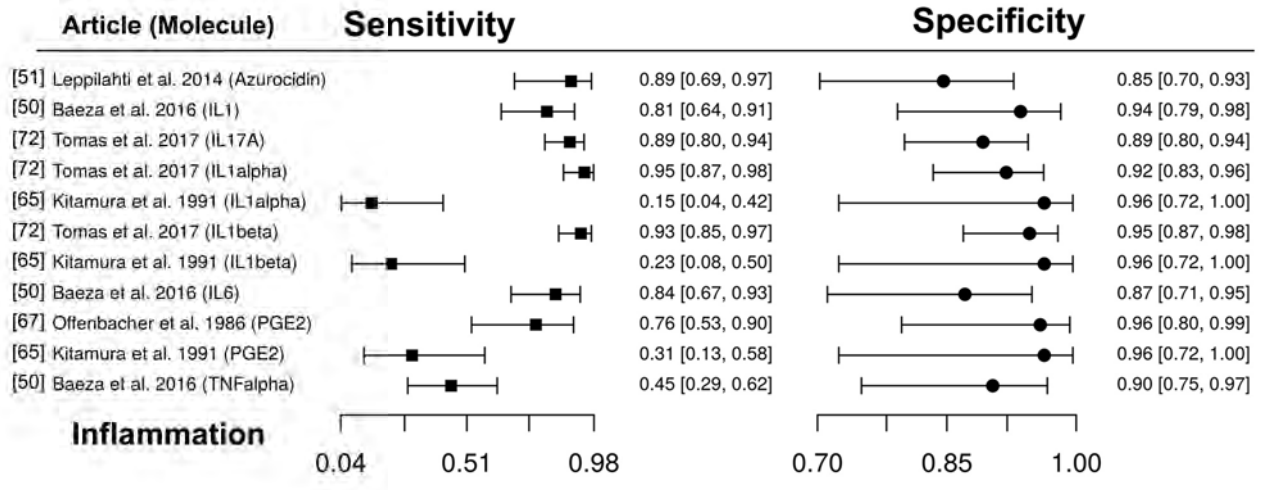


Figure 6C. Coupled forest plot with diagnostic test accuracy (sensitivity, specificity, and 95% confidence interval) of the classifications of the inflammation molecules included only in the qualitative analysis. The biomarkers included in the qualitative analysis were those that presented less than three classifications in less than three articles. In the coupled forest plot, sensitivity and specificity values are rounded.

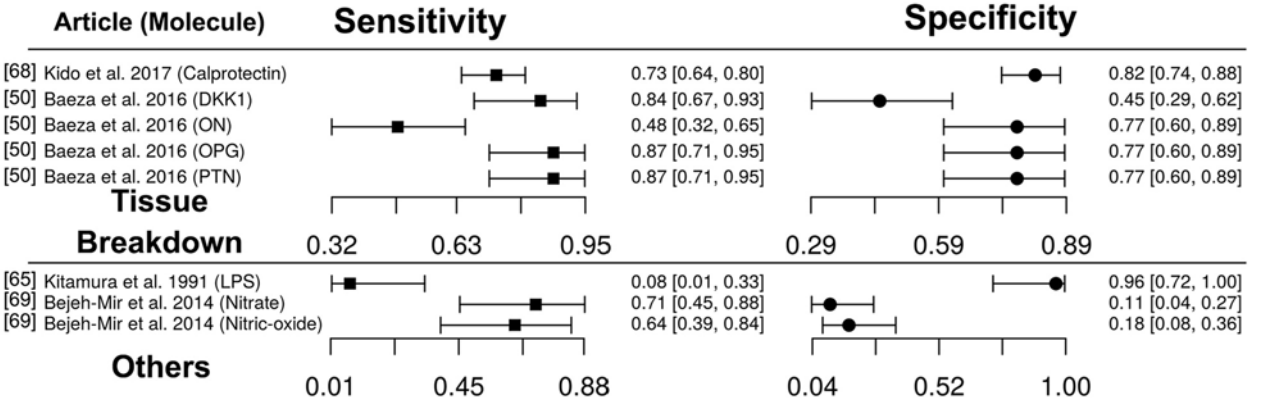


Figure 6D. Coupled forest plot with diagnostic test accuracy (sensitivity, specificity, and 95% confidence interval) of the classifications of the tissue breakdown products and other molecules included only in the qualitative analysis. The biomarkers included in the qualitative analysis were those that presented less than three classifications in less than three articles. In the coupled forest plot, sensitivity and specificity values are rounded.

Table 7. Diagnostic test accuracy measurements for each GCF biomarker classification included only in the qualitative analysis.

Study (first author)	Index Test		No. CC/ No. TC	Threshold (Units)	AUC	ACC (%)	SENS/ SPEC (%)	PPV/ NPV (%)	LR+/ LR-	DOR	J Index
	Name	Type									
Nakashima [61]	ALP	ENZYMES	36/ 37	1300.0 (µIU/30 s)	NSp	75.5	30.0/ 86.0	33.3/ 84.1	2.2/ 0.8	2.6	0.16
Chapple [62]	ALP		43/ 10	115.5 (µU/sample)	NSp	75.3	78.4/ 72.2	74.4/ 76.5	2.8/ 0.3	9.4	0.51
Rutger [63]	AST		178/ 22	800.0 (µIU)	NSp	70.5	90.9/ 68.0	26.0/ 98.4	2.8/ 0.1	21.2	0.59
Ito [58]	AST		205/ 78	877.2 (µU)	0.682	64.3	73.1/ 61.0	41.6/ 85.6	1.9/ 0.4	4.2	0.34
Nakashima [61]	BG		35/ 24	2; 4 (U)	NSp	81.1	30.0/ 93.0	50.0/ 85.1	4.3/ 0.8	5.7	0.23
Lamster [64]	BG		43/ 10	444.6 (mU/sample)	NSp	88.1	91.7/ 85.7	81.5/ 93.8	6.4/ 0.1	66.0	0.77
Nakashima [61]	COL		43/ 10	1395.6 (mU/sample)	NSp	81.1	30.0/ 93.0	50.0/ 85.1	4.3/ 0.8	5.7	0.23
Kitamura [65]	COL		13/ 13	NSp (NSp)	NSp	56.6	15.4/ 96.3	80.0/ 54.2	4.2/ 0.9	4.7	0.12
Kitamura [65]	COL		13/ 13	NSp (NSp)	NSp	52.8	7.7/ 96.3	66.7/ 52.0	2.1/ 1.0	2.2	0.04
Kitamura [65]	COL		13/ 13	NSp (NSp)	NSp	52.8	7.7/ 96.3	66.7/ 52.0	2.1/ 1.0	2.2	0.04
Eley [57]	DPP		288/ 240	3.5 (µU/60 s)	NSp	72.0	82.9/ 62.8	65.0/ 81.5	2.2/ 0.3	8.2	0.46
Eley [56]	DPP		238/ 290	3.5 (µU/60 s)	NSp	79.9	79.0/ 81.1	83.6/ 76.0	4.2/ 0.3	16.1	0.60

Leppilahti [51]	MMP13	39/ 19	2.69 (ng/ml)	0.94	87.9	89.5/ 87.2	77.3/ 94.4	7.0/ 0.1	57.8	0.77
Leppilahti [51]	MMP14	39/ 19	357.8 (ng/ml)	0.950	91.4	94.7/ 89.7	81.8/ 97.2	9.2/ 0.1	157.5	0.85
Baeza [50]	MMP9	31/ 31	514.0 (au/ml)	0.910	88.7	96.8/ 80.6	83.3/ 96.2	5.0/ 0.0	125.0	0.77
Baeza [50]	MPO	19/ 19	2520.6 (ng/ml)	0.900	85.5	93.5/ 77.4	80.6/ 92.3	4.1/ 0.1	49.7	0.71
Leppilahti [51]	MPO	31/ 31	7.9 (ng/ml)	0.980	94.7	94.7/ 94.7	94.7/ 94.7	18.0/ 0.1	324.0	0.90
Baeza [50]	ProMMP2	31/ 31	28.5 (au/ml)	0.990	95.2	93.5/ 96.8	96.7/ 93.8	29.0/ 0.1	435.0	0.90
Baeza [50]	ProMMP9	31/ 31	1275.0 (au/ml)	1.000	95.2	96.8/ 93.5	93.8/ 96.7	15.0/ 0.0	435.0	0.90
Hemmings [66]	Protease	38/ 92	NSp (score)	NSp	80.0	88.0/ 60.5	84.4/ 67.6	2.2/ 0.2	11.3	0.49
Gul [53]	Sialidase	30/ 60	2.2 (NS)	0.790	79.2	79.2/ 79.2	88.4/ 65.6	3.8/ 0.3	14.5	0.58
Gul [52]	Sialidase	77/ 154	2.3 (ng/ul)	0.880	83.3	86.7/ 76.7	88.1/ 74.2	3.7/ 0.2	21.4	0.63
Ito [58]	T-Protein	205/ 78	16.8 (µg/0.5 ml)	0.810	77.0	75.6/ 77.6	56.2/ 89.3	3.4/ 0.3	10.7	0.53
Baeza [50]	TRAP5	31/ 31	0.5 (pg/ml)	0.980	91.9	93.5/ 90.3	90.6/ 93.3	9.7/ 0.1	135.3	0.84
Eley [57]	Tryptase	238/ 290	1.8 (µU/60 s)	NSp	64.2	67.9/ 61.1	59.3/ 69.6	1.7/ 0.5	3.3	0.29
Eley [56]	Tryptase	288/ 240	1.8 (µU/60 s)	NSp	68.0	69.0/ 66.8	71.7/ 63.9	2.1/ 0.5	4.5	0.36

Leppilahti [51]	Azurocidin	INFLAMMATION	39/19	299.3 (pg/ml)	0.900	86.2	89.5/84.6	73.9/94.3	5.8/0.1	46.8	0.74
Baeza [50]	IL1		31/31	5.9 (pg/ml)	0.920	87.1	80.6/93.5	92.6/82.9	12.5/0.2	60.4	0.74
Tomás [72]	IL17A		74/73	NSp (pg/ml)	0.937	89.1	89.0/89.2	89.0/89.2	8.2/0.1	67.0	0.78
Tomás [72]	IL1alpha		74/73	NSp (pg/ml)	0.973	93.2	94.5/91.9	92.0/94.4	11.7/0.1	195.5	0.86
Kitamura [65]	IL1alpha		13/13	NSp (NSp)	NSp	56.6	15.4/96.3	80.0/54.2	4.2/0.9	4.7	0.12
Tomás [72]	IL1beta		74/73	NSp (pg/ml)	0.963	93.9	93.2/94.6	94.4/93.3	17.2/0.1	238.0	0.88
Kitamura [65]	IL1beta		13/13	NSp (NSp)	NSp	60.4	23.1/96.3	85.7/56.5	6.2/0.8	7.8	0.19
Baeza [50]	IL6		31/31	1.6 (pg/ml)	0.930	85.5	83.9/87.1	86.7/84.4	6.5 /0.2	35.1	0.71
Offenbacher [67]	PGE2		24/17	66.2 (ng/ml)	NSp	87.8	76.5/95.8	92.9/85.2	18.4/0.2	74.8	0.72
Kitamura [65]	PGE2		13/13	NSp (pg/µl)	NSp	64.2	30.8/96.3	88.9/59.1	8.3/0.7	11.6	0.27
Baeza [50]	TNFalpha		31/31	1.2 (pg/ml)	0.640	67.7	45.2/90.3	82.4/62.2	4.7/0.6	7.7	0.36

Kido [68]	Calprotectin	TISSUE	120/118	17.2 (µg/ml)	0.826	77.3	72.9/81.7	79.6/75.4	4.0/0.3	12.0	0.55
Baeza [50]	DKK1		31/31	0.004 (pg/ml)	0.730	64.5	83.9/45.2	60.5/73.7	1.5/0.4	4.3	0.29
Baeza [50]	ON		31/31	0.710 (pg/ml)	0.760	62.9	48.4/77.4	68.2/60.0	2.1/0.7	3.2	0.26
Baeza [50]	OPG		31/31	0.030 (pg/ml)	0.910	82.3	87.1/77.4	79.4/85.7	3.9/0.2	23.1	0.65
Baeza [50]	PTN		31/31	0.019 (pg/ml)	0.950	82.3	87.1/77.4	79.4/85.7	3.9/0.2	23.1	0.65
Kitamura [65]	LPS	OTHERS	13/13	NSp (NSp)	NSp	52.8	7.7/96.3	66.7/52.0	2.1/1.0	2.2	0.04
Bejeh-Mir [69]	Nitrate		28/14	4.970 (NSp)	0.270	31.0	71.4/10.7	28.6/42.9	0.8/2.7	0.3	-0.18
Bejeh-Mir [69]	Nitric oxide		28/14	10.120 (NSp)	0.280	33.3	64.3/17.9	28.1/50.0	0.8/2.0	0.4	-0.18

The biomarkers included in the qualitative analysis were those that presented less than three classifications in less than three articles. Biomarkers were listed in alphabetical order within each type of biomarker.

1.5. DISCUSSION

1.5.1. Quality of Diagnostic Accuracy Studies in GCF and Heterogeneity Observed

The application of a dual process (computerised and manual) for the selection of articles enabled us to ascertain that the diagnostic accuracy literature on GCF molecular biomarkers in periodontitis represents less than 2% of the global search. Subsequently, we found that 38% of the diagnostic accuracy studies in GCF detected did not meet the methodological requirements for inclusion in a systematic review on diagnostic accuracy. These essential requirements are the description of sensitivity and specificity values or, failing that, sufficient data to calculate the corresponding 2x2 contingency table [49]. To the best of our knowledge, the present study is the first systematic review/meta-analysis of accuracy studies on GCF biomarkers for the diagnosis of periodontitis.

In terms of the methodological quality of the papers assessed using the QUADAS-2 tool [33], a predominance of case-control studies was detected (around 75%), which are known to be at higher risk of bias than cohort studies [40]. However, it is important to emphasise a very positive characteristic present in practically all the included articles, which is the presence of a “single-gate” design. A "single gate" study is considered when three premises are fulfilled: 1) a single set of inclusion criteria is used for recruiting patients in the same setting; 2) to verify who has the disease and who does not, all patients undergo the reference standard; 3) all patients, reference standard-positives and reference standard-negatives, undergo the index test(s) [13,37]. Accordingly, unlike in other diseases where the reference standard can only verify the diagnosis in a group of study subjects (partial verification) [70], the total verification feature present in the papers on GCF biomarkers in periodontitis contributes to controlling possible imbalances between sensitivity and specificity values [37]. The studies with “single-gate” design reflect reality better than the “two-gate” studies and are more likely to provide valid estimates of diagnostic accuracy [37] controlling possible imbalances between sensitivity and specificity values [37,70].

On the contrary, a predominant negative aspect associated with a high risk of bias in the index test domain is how the selection of the threshold takes place. In the present review, the authors did not apply pre-specified thresholds of the GCF molecular biomarkers in about 80% of the articles but instead selected the threshold to optimise sensitivity and specificity. This way of proceeding undoubtedly leads to an overestimation of the performance of the test [33]. Although the threshold optimisation is a choice that can be adopted in the initial stages of the discovery of diagnostic biomarkers, a subsequent validation analysis in the article itself, whether internal or external, is fundamental when it comes to achieving robust results [71]. However, of all the articles included in the present review, only the series recently published by Tomás et al. [72] included an internal validation analysis of the performance results of index tests (in this case, a set of GCF cytokines). This fact is also the case in the paper by Gul et al. [53], who carried out an external validation of the index tests against independent data (in this case, MMP8, elastase and sialidase). On the other hand, recently Gürsoy et al. [73] proposed the cumulative use of salivary biomarkers with an adaptive-threshold design as an alternative to fixed biomarker thresholds in the detection of periodontitis.

In our opinion, there is a fundamental matter to consider in the methodological quality of diagnostic accuracy articles that is not contemplated in the QUADAS-2 tool, namely the sample size of the control and target groups used to produce the diagnostic classification table. This premise is the main methodological limitation identified in the included studies, since about 84% of the included classifications had ≤30 subjects in each group (control and target condition), while only 12.0% had >70 subjects. Fortunately, these data improved significantly when the analyses were carried out at the site level, although still, 28% of the classifications had ≤30 subgingival sites. The performance results of an index test derived from a group of ≤30 subjects/sites in the control or target condition are unreliable because the variation in the classification of a single subject/site causes a modification of >3.3% in the sensitivity or specificity values. We believe that this requirement is of utmost importance in the methodological quality of a diagnostic

accuracy study, and so its inclusion in the items of the QUADAS-2 tool should be reconsidered.

Concerning the observed heterogeneity, a distinction could be made between aspects related to the reference standard (clinical and radiological parameters) for the establishment of the control and target conditions, and those related to the detection of molecular biomarkers in GCF. Regarding the first issue, as there are no universally accepted clinical diagnostic criteria for defining what a periodontitis case is [74], the variability of the definitions of the clinical phenotype of periodontitis in diagnostic accuracy studies had to be accepted to perform this systematic review.

From a methodological point of view, the reference standard for the diagnosis of the periodontal condition based on only clinical (PPD or CAL) or clinical and radiographic parameters (BL) was considered the “gold standard”. This premise means that GCF biomarker accuracy estimates are calculated under the theoretical assumption that the reference standard is 100% sensitive and specific [40,70]. It is therefore impossible to show that a GCF biomarker is better than the clinical and radiographic parameters, even if this would be the case in reality [37]. However, the well-recognised imprecision of the parameters described above (error-prone measures) and the application of heterogeneous clinical diagnostic criteria contribute to the fact that the reference standard is not "perfect" [74,75]; it can potentially lead to errors in the estimation of the diagnostic accuracy of GCF biomarkers [40]. In the case of periodontitis, we believe that this possible verification bias will tend to underestimate biomarker accuracy since the reference standard and the index test both measure different aspects of the disease (clinical and biological phenotypes), meaning that their possible errors are unrelated [70].

Periodontal disease progression is episodic on a tooth site-level; however, the risk of developing the periodontal disease is principally on the patient level [76]. Assuming this dual perspective [17], in the included series, the accuracy analysis at site level predominated (79% *versus* 21% at patient-level).

It has been stated that reported estimates of diagnostic accuracy may have limited clinical applicability (generalisability) if the spectrum of tested patients is not similar to the patients who would undergo the biomarker test in practice. Studies that include severe cases and healthy controls tend to overestimate diagnostic performance [40,70]. Accordingly, in order to include a broad spectrum of patients for the GCF biomarkers under investigation, and to counteract the risk of performance overestimations, this review considered different control (e.g., subjects with good periodontal health or those with good periodontal health and gingivitis) and target conditions (e.g., different degrees of the extent and severity of chronic periodontitis).

Concerning other heterogeneous aspects related to the detection of GCF molecular biomarkers [10], we observed differences in methodological aspects of GCF collection. It is important to note that very low GCF volumes can have a dramatic effect on the concentrations of GCF biomarkers [11] and, consequently, on their diagnostic accuracy. It is also evident that the GCF collection protocol and the type of technique and the methodological protocol applied can vary the detection and quantification capacity of biomarkers [77]. In the present study, the most frequently used techniques were colourimetric or fluorimetric methods, followed by multiparametric cytometry and the ELISA techniques. Another consideration to be taken into account is how the manipulation of the data below the detection limit of the techniques was carried out [78]; an aspect that was not clarified in most of the papers included in the present systematic review.

One of the most important sources of heterogeneity between the diagnostic accuracy studies is a “threshold effect” [44]. In the studies that analysed the biomarkers that were subjected to a meta-analytic analysis, the authors used different thresholds. In the present study, however, no threshold effect was detected that would condition the performance values of these biomarkers. The exception was trypsin, and the explanation for this lies in the fact that three of the four classifications came from the same article.

1.5.2. Accuracy of Biomarkers in GCF for the Diagnosis of Periodontitis

As we have not identified any systematic review/meta-analysis of GCF biomarkers for the diagnosis of periodontitis, our results cannot be compared to other similar meta-analytical papers. Indeed, we were only able to discuss our findings by comparing them with those in reviews that are not focused on diagnostic accuracy and other research papers of interest.

The first important observation to make is that there is a large number of individual biomarkers of a different nature, with up to 36 that have been evaluated from an accuracy perspective. The biomarkers are of different types, covering the three principal biological phases of periodontitis (inflammatory, connective tissue degradation and bone-turnover) [79]. However, most of these observations are only described in a single article and are therefore unreliable, while only 4 (11%) were analysed in at least three publications. Our impression is that there is scientific attention about the initial discovery of GCF biomarkers for the diagnosis of periodontitis. There is, however, a lack of interest in confirming initial accuracy results, even though this is necessary if a biomarker is to be considered reliable. Based on the findings of this review, enzymes are, undoubtedly, the most researched biomarkers in the accuracy field, with the most studied of these being MMP8, followed by elastase, cathepsin and trypsin. This discovery enabled their quantitative analysis in the present review.

MMPs are key proteases involved in periodontitis and are associated with periodontal status. MMP8 is the main collagenase in the disease: 90% to 95% of collagenolytic activity in GCF originates from MMP8 [80]. In 2005, in an excellent review of host-derived diagnostic biomarkers for periodontitis by Loos and Tjoa [81], MMP8 was shown to be a promising, but not a definitive, biomarker for distinguishing periodontitis from periodontal health and gingivitis. These authors noted that MMP8 is a complicated molecule, and its implication in disease is not evident because it has a latent and an active form.

From 2005 to date, there has been a significant increase in the number of studies that demonstrate both the association between MMP8 and various periodontal diseases and its high predictive capability. Indeed, some researchers have even commercialised several kits for the diagnosis of periodontitis and peri-implantitis based on MMP8 [80]. In a comprehensive review published by Sorsa et al. in 2016 [80], these authors stated: "antibodies originally described by themselves and used in Periomarker®, Periosafe®, Implantsafe® and Oral Risk Indicator® tests showed sensitivities of 0.83 and 0.95 and specificities of 0.96 and 0.98 for rapid point-of-care/chair-side and quantitative laboratory tests, respectively". These authors support these accuracy data by referencing two papers: one conducted by Leppilahti et al. in 2014 [51]; and one by Mäntylä et al. in 2003 [55].

In the present meta-analysis, MMP8 was the molecule associated with the best performance values, since it showed an estimated sensitivity and specificity median of 77% and 92%, respectively. However, unlike Sorsa et al. [80], it was precisely the results of the previously cited paper by Mäntylä et al. [55], which was included in our meta-analysis, that provided the worst sensitivity data (64% and 70%), negatively conditioning the estimation of the parameter.

Neutrophil elastase is an abundant proteinase released from the azurophilic granules of neutrophils; as a consequence, it is an indicator of neutrophil activity [82]. In line with the conclusions reached by Loos and Tjoa [81] about the promising diagnostic capability of elastase, the present review found that this molecule was the second most researched and had the second-best performance values (estimated sensitivity and specificity median=75% and 81%). Cathepsin and trypsin were the other two biomarkers that faced a meta-analysis. These molecules had lower test performance values, with trypsin being the worst biomarker, with sensitivity and specificity percentages below 70%.

Our results should be interpreted with caution concerning other less researched GCF biomarkers. However, it is important to highlight the accuracy data of five biomarkers derived from the series where >30 subjects/GCF samples were evaluated: myeloperoxidase (MPO),

IL1alpha, IL1beta, IL17A and IL6. In contrast to what was found by Loos and Tjoa in 2005 [81], Baeza et al. in 2016 [50] demonstrated that the enzyme MPO had outstanding sensitivity and specificity values for the diagnosis of periodontitis (95% and 94%).

Several authors have stated that markers of inflammation are not specific enough to detect the development of periodontitis [74], and could, therefore, be of limited use [83]. Interestingly, however, the authors of several systemic reviews agreed in observing a distinctive profile of specific cytokines in patients with chronic periodontitis [23,84]. In line with these findings, in a paper included in the present review and published by Tomás et al. [72], the authors found excellent sensitivity and specificity values for both IL1 alpha and IL1beta (95% and 92%, and 93% and 95%, respectively). These same authors [72] and Baeza et al. [50] also observed that other pro-inflammatory cytokines such as IL17A and IL6 produce excellent performance measures (sensitivity=89% and 84%, respectively; specificity=89% and 87%, respectively).

In terms of the influence of variables on the diagnostic accuracy of GCF biomarkers, we are especially interested in the smoking condition. Despite the inclusion of both non-smokers and smokers in some of the series examined in the present review, only one study took this variable into account, adjusting its predictive models and performance measures to this clinical variable [72]. However, there are several studies that have revealed that enzymes such as MMP8, elastase and MPO, or inflammatory mediators such as cytokines, have different profiles in the expressions or levels of these biomarkers in gingival tissue or GCF samples in periodontal smokers and non-smokers [85-88]. These findings confirm the immunosuppressive effect of smoking, which could significantly affect the accuracy of biomarkers for the diagnosis of periodontitis. Consequently, the smoking condition should be considered in future designs of diagnostic accuracy studies.

1.5.3. Implications for Practice

It has been stated that the biologic phenotype of periodontitis is not reflected correctly in the clinical phenotype [89]. If the biological phenotype could be evaluated in clinical practice through the determination of GCF biomarkers, this would help to improve the diagnosis of periodontitis. In this sense, in the recently published, new classification of Periodontal and Peri-implant Diseases and Conditions, Tonetti et al. [6] recognised that biomarkers might contribute to improved diagnostic accuracy in the early detection of periodontitis and any assessment of its severity grade. This consideration is so accepted that the proposed classification framework allows the introduction of validated biomarkers in the case definition system.

Biomarkers indicate health, disease and response to therapy and must also be robust and proved to be valid in clinical studies [11]. However, the natural progression of periodontitis substantially complicates the discovery of GCF biomarkers with diagnostic capability, as the periodontal disease progresses episodically, with the challenge being to define quiescent and active periods [15]. This fact is reflected in the present review because, although numerous biomarkers were evaluated, the reality is that only two, MMP8 and elastase, have been investigated by different researchers in different series. Consequently, these could be regarded as being the most reliable when it comes to their clinical applicability.

An ideal diagnostic test should, of course, have sensitivity and specificity values approaching 100%. Unfortunately, however, this is never the case [17]. However, a first-line test, also called a triage test, may be clinically useful even when the sensitivity or specificity is not high, depending on the steps that will be taken after testing [37].

Considering the interpretation of sensitivity and specificity used by some authors [46], MMP8 was a better biomarker than elastase, as it showed a good capability to distinguish patients with periodontitis and an excellent capability to distinguish those without the disease. In terms of usefulness or effectiveness of the biomarker, considering a 45%

prevalence of periodontitis associated with a broad spectrum of disease [59,60], theoretically, MMP8 test would be more clinically useful or effective than elastase test, showing almost 90% true positives and around 83% true negatives. If we consider that MMP8 test in GCF was used as a first-line test to decide who should be referred for additional testing [37], there would be 17% of periodontitis subjects where the test would not be able to detect disease in initial screening and on the other hand, only 11% of patients would be undergoing an unnecessary periodontal exploration.

1.5.4. Strengths and Limitations

Diagnostic accuracy studies are described in different ways, and there is no standard terminology available [37]. Following the recommendations of the Cochrane group [90], we avoided using search filters based on methodological terms to identify diagnostic accuracy studies, thereby reducing the likelihood of missing articles on the topic. Given the magnitude of the search, the use of a dual process (computerised and manual) for the selection of articles was mandatory. The dual search and selection process guaranteed that the articles included in our study accounted for the vast majority of all relevant diagnostic accuracy papers and that the final results were significant.

Due to a large amount of data that had to be extracted from the included papers, two independent reviewers focused on the accuracy analysis data and two other reviewers on the characteristics of the studies; then roles were exchanged to review the extracted data. This approach decreased the risk of error and subjectivity to a minimum.

We deliberately chose to include a broad spectrum of health and periodontitis in order to provide more realistic accuracy estimates for GCF biomarkers [70]. We did not assess publication bias because no accurate reporting methods for diagnostic accuracy studies exist [37].

We did not perform tests to evaluate publication bias, as these can be misleading when they are applied to systematic reviews of diagnostic test accuracy [37,91,92].

The accuracy of a systematic review depends on the quantity and quality of the studies included. One of the main limitations of this review was that it did not have a higher number of series with large sample sizes in order to obtain more robust results from meta-analyses. Similarly, the limited number of classifications included for each biomarker did not allow us to perform analysis for heterogeneity or sensitivity analyses with which to evaluate the influence of covariates as the type of technique used for the quantification of biomarkers.

Another general limitation in diagnostic accuracy reviews is that biomarkers analysed in different studies are compared. Comparisons between biomarkers should be ideally performed in the same series, in the same patients and against the same reference standard [93].

1.5.5. Implications for Research: Future Perspectives

Despite being a subject of great interest to the scientific community, in the current literature on the diagnostic accuracy of single molecular biomarkers in GCF, there is a predominance of individual results from a multitude of molecules; with MMP8 and elastase being the most researched biomarkers. On the other hand, a considerable number of studies are of questionable quality, especially involving series with small sample sizes.

It is, therefore, necessary to perform sufficiently large, prospective, well-designed, multicentre studies that evaluate several GCF biomarkers for diagnosis of periodontitis (or variations on a test) [40]. Also, these studies have to include a validation analysis, at least internal validation [71], and should also consider the influence of variables such as the presence of gingivitis in the control condition, smoking and the presence of systemic diseases in order to refine the diagnostic accuracy results. This increase in high-quality evidence would allow other more exhaustive meta-analyses to be conducted, including threshold and heterogeneity analyses [49,94].

Assuming the importance of the concept “biological signature” [19], a systematic review to both identify the set of biomarkers with the most favourable combination of sensitivity and specificity and assess

the feasibility of their clinical application would be another exciting contribution to the field of diagnostic accuracy.

It is also important to continue meta-analytically investigating other essential properties of biomarkers, such as their prognostic capacity for disease progression or their ability to predict response to treatment. It should also be noted that the "time" factor plays a fundamental role in any analysis of these studies [95].

In conclusion, MMP8 in GCF showed good sensitivity and excellent specificity resulting in the clinically most useful or effective biomarker for a broad spectrum of disease in systemically healthy subjects. Other molecules, such as MPO or several pro-inflammatory cytokines, were identified as promising GCF biomarkers for the diagnosis of periodontitis, but more high-quality research is required to confirm these observations.

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OBJECTIVE 2

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Objective 2. How Accurate Are Single Molecular Biomarkers in Saliva for the Diagnosis of Periodontitis? A Systematic Review and Meta-Analysis

2.1. ABSTRACT

Aim: To analyse, using a meta-analytical approach, the diagnostic capacity of molecular biomarkers in saliva for the detection of periodontitis in systemically healthy subjects.

Material and Methods: Articles on molecular biomarkers in saliva providing a binary contingency table (or failing that, sensitivity and specificity values and group sample sizes) in individuals with clinically diagnosed periodontitis were considered eligible. Searches for candidate articles were conducted in six electronic databases. The methodological quality of the included studies was assessed through the tool quality assessment of diagnostic studies (QUADAS-2). Meta-analyses were performed using the hierarchical summary receiver operating characteristic (HSROC) modelling, which adjusts study classification data using random-effects logistic regression.

Results: Meta-analysis was possible for 5 of the 32 biomarkers studied. The highest values of sensitivity for the diagnosis of periodontitis were obtained for IL1beta (78.7%), followed by MMP8 (72.5%), IL6 and Hb (72.0% for both molecules); the lowest sensitivity value was for MMP9 (70.3%). In terms of specificity estimates, MMP9 had the best result (81.5%), followed by IL1beta (78.0%) and Hb (75.2%); MMP8 had the lowest specificity (70.5%).

Conclusions: MMP8, MMP9, IL1beta, IL6 and Hb were salivary biomarkers with good capability to detect periodontitis in systemically

healthy subjects. MMP8 and IL1beta were the most researched biomarkers in the field, both showing clinically fair effectiveness for the diagnosis of periodontitis.

Clinical Relevance: The scientific community is increasingly promoting the discovery of objectively quantifiable biomarkers in saliva that can reliably reflect the physiopathological condition of the gingival sulcus and improve the early detection and monitoring of periodontitis. MMP8 and IL1beta were the most researched salivary biomarkers in the diagnostic accuracy field, both showing good capability to detect periodontitis; although IL1beta showed higher estimated values of sensitivity and specificity. Considering its performance results, IL1beta and MMP8 showed clinically fair effectiveness for the diagnosis of periodontitis in systemically healthy subjects.

2.1.1. Keywords

Systematic review; meta-analysis; diagnostic accuracy; molecular biomarkers; saliva; periodontitis.

2.1.2. Declaration of Conflict of Interest

The doctoral candidate and the rest of the authors declare that they have no conflict of interest concerning the objectives proposed in this chapter.

2.1.3. Funding

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2.2. Introduction

2.2.1. Target Condition Being Diagnosed

An estimated 743 million people are affected by periodontitis, which is considered to be the sixth most prevalent disease globally [1]. A new classification of Periodontal and Peri-implant Diseases and Conditions has recently been established. This classification recognises the existence of three different clinical entities: periodontitis (including the types previously defined as “chronic” or “aggressive”); necrotising periodontitis; and periodontitis as a manifestation of systemic disease. This classification framework is based on a multidimensional staging and grading system [2,3], the characteristics of which have been detailed in Objective 1. A periodontitis patient remains so for life, even following successful therapy, and requires life-long supportive care to prevent any recurrence of the disease [4].

2.2.2. Index Test(s)

Biomarkers provide a measurable "signature" of health status and disease [5]. It is rightly said that saliva is the mirror of the body and, due to the non-invasive character of saliva collection, this fluid is considered to be useful for screening tests and the monitoring of periodontitis [6]. In the present systematic review, and as in Objective 1, the index tests evaluated were molecular biomarkers detected in saliva derived from both the host and the microbiota.

It is essential to distinguish the three types of accuracy studies - diagnostic, prognostic and predictive [7] - that can be developed in the field in order to determine the characteristics of a biomarker [8].

2.2.3. Clinical Pathway

In Periodontics, the diagnosis of periodontitis is a crucial element in the success of treatment, as the progression of the disease causes an irreversible loss of periodontal structures [9]. The traditional clinical and radiographic parameters are the best measures currently available for diagnosing the disease and monitoring its progress, potential treatment and maintenance [3]. Nevertheless, these parameters can only

assess the previous episodes and severity of periodontitis, and no reliable information can be obtained regarding the current activity of the disease and its future course. Furthermore, the episodic progression of periodontitis makes an accurate assessment of the disease status difficult and complicated [10]. Accordingly, clinical monitoring is time-consuming, subject to considerable measurement error and is often poorly tolerated by patients [6].

As a consequence, the scientific community is increasingly promoting the discovery of objectively quantifiable biomarkers in saliva that can reliably reflect the physiopathological condition of the gingival sulcus and improve the early detection and monitoring of periodontitis [11-13].

2.2.4. Rationale

Several systematic reviews have revealed the role of salivary biomarkers such as matrix metalloproteinase (MMP) 8, interleukin (IL) 1beta and tumour necrosis factor (TNF) alpha in the pathogenesis and progression of the disease [14,15].

However, research on the diagnostic capability of a biomarker requires the design of a specific accuracy study. This type of study provides estimates of the test performance [16], as sensitivity and specificity, whose determination constitutes essential requirements of a useful diagnostic biomarker [17]. We have identified only one systematic review on the accuracy of salivary biomarkers for the diagnosis of periodontitis, but it does not provide meta-analytical results for any specific biomarker [18].

Consequently, the objective of this systematic review/meta-analysis was to examine the literature to determine the accuracy of single molecular biomarkers detected in saliva for diagnosing periodontitis in systemically healthy patients.

2.3. Material and Methods

This systematic review/meta-analysis was written according to the Cochrane handbook for systematic reviews of diagnostic test accuracy, version 1.0.0 [19] and the PRISMA-DTA statement [16]. The completed PRISMA-DTA checklist is provided in Appendix S1.

The protocol was registered at the International Prospective Register of Systematic Reviews (PROSPERO) under the number CDR 42019124428.

2.3.1. PICO Question

The formulated PICO question (patient, index test, comparison, outcome) was as follows: "In systemically healthy subjects with periodontitis, does the expression of individual molecular biomarkers in saliva exhibit diagnostic capability of periodontitis in comparison to conventional clinical parameters?"

2.3.2. Criteria for Considering Studies for this Review

2.3.2.1. Types of Accuracy Study

Studies (whether cross-sectional, longitudinal or interventional) on molecular biomarkers (index tests) in saliva that showed results on diagnostic accuracy in individuals with clinically diagnosed periodontitis (reference standard) were eligible for inclusion. Excluded were studies that did not report: 1) a contingency table for binary classification (2x2 table that includes a number of: true positives, true negatives, false positives and false negatives); or 2) sensitivity and specificity values and sample sizes of the control and target groups, from which the calculation of classification tables could be possible.

2.3.2.2. Participants

The subjects included in this review were patients without an explicit diagnosis of systemic disease and with a clinical periodontal diagnosis. Studies on patients with clearly defined syndromes or systemic diseases/conditions, as well as those on animal experimentation or *in vitro* models, were excluded.

2.3.2.3. Control and Target Conditions

According to the classification of periodontal diseases and conditions published by Armitage [20], the target conditions considered were chronic and aggressive periodontitis, regardless of the extent of the disease and the degree of severity. Studies whose target conditions were gingivitis, peri-implantitis or periodontal conditions other than chronic or aggressive periodontitis were excluded. For the control condition, patients with a clinical diagnosis of periodontal health and gingivitis were considered.

2.3.2.4. Reference Standard

The reference standard for the diagnosis of a periodontal condition was based on only clinical parameters (probing pocket depth -PPD- or clinical attachment loss -CAL-) or clinical and radiographic parameters (bone loss -BL-), irrespective of the diagnostic criteria applied. Consequently, in the absence of homogeneous criteria, any definition based on the author's reported criteria was accepted. A clinical periodontal diagnosis is a binary aspect of two categories, control and target condition, which is established at the patient-level (e.g., patients with periodontal health *versus* those with chronic periodontitis).

Studies that did not detail any reference standard for the diagnosis of the periodontal condition were ineligible for inclusion in this review.

2.3.2.5. Index Test(s)

Any single molecular biomarker detected in saliva, which was analysed from an accuracy analysis perspective, was considered to be an index test. Accuracy studies on multi-biomarkers or those detected in other fluids (e.g., blood) were excluded.

2.3.2.6. Other Exclusion Criteria

The following types of study were excluded: theses, dissertations, reviews, letters, personal opinions, book chapters, short communications, conference abstracts and patents. Other considerations applied were: (i) no restrictions on the publication date of the papers, the type of setting or the publication status; and (ii) the articles had to be written in English.

2.3.3. Search Methods for the Identification and Selection of Studies

2.3.3.1. Information Sources and Search Strategy

The search was conducted through the following electronic databases: Pubmed (Medline), Embase, the Cochrane Central Register of Controlled Trials and Trial Protocols, Scopus, Lilacs, and Web of Sciences (WoS).

Following the recommendations established by the Cochrane Group for Systematic Reviews of Diagnostic Test Accuracy [19], the search strategy to identify accuracy studies involved three sets of terms: 1) terms to search for the target condition (periodontitis); 2) terms to identify the index tests (molecular biomarkers) under evaluation; 3) terms to establish the type of oral sample analysed (saliva). In order to decrease the loss of any relevant studies, any search filter based on methodological terms was avoided. Checks of the references of the included studies and other relevant reviews on the topic were also performed. The search strategy used in the different electronic databases was performed on october 25th, 2018, and is detailed in Table 1.

Table 1. Search strategy, which was applied in the different databases.

• **TERMS FOR TARGET CONDITION**		
1. Periodontitis 2. Periodontal 3. 1 or 2 AND		
• **TERMS FOR THE TYPE OF ORAL SAMPLE ANALYSED**		
4. Saliva AND		
• **TERMS FOR THE INDEX TESTS**		
5. Amino acid 6. Antibody 7. Enzyme 8. Immunoglobulin 9. Marker 10. Mediator 11. Metabolite 12. Peptide 13. Protein 14. Substance	15. Activating factor 16. Adipocytokine 17. Adiponectin 18. Albumin 19. Aminopeptidase 20. Aminotransferase 21. Amylase 22. Antitrypsin 23. Arginase 24. Arylsulfatase 25. Ascorbate 26. Calcium 27. Calgranulin 28. Calprotectin 29. Cathepsin 30. CD14 31. Chemokine 32. Chitinase 33. Chondroitin 34. Collagenase 35. Complement C 36. Cortisol 37. Creatine 38. Creatinine 39. Cystatin 40. Cytokine 41. Dehydrogenase	54. Hydroxyproline 55. Interferon 56. Interleukin 57. Keratin 58. Lactoferrin 59. Laminin 60. Leptin 61. Leukotriene 62. Lysozyme 63. Macroglobulin 64. Melatonin 65. Metalloproteinase 66. Microglobulin 67. Myeloperoxidase 68. Neopterin 69. Neurokinin 70. Nitrate 71. Nitric oxide 72. Nitrite 73. Osteocalcin 74. Osteonectin 75. Osteopontin 76. Osteoprotegerin 77. Peptidase 78. Peroxidase 79. Phosphatase 80. Plasminogen

	42. Dipeptidylpeptidase	81. Prostaglandin
	43. Elastase	82. Protease
	44. Esterase	83. Proteinase
	45. Fibronectin	84. Pyridinoline
	46. Gingipain	85. RANKL
	47. Glucuronidase	86. RANTES
	48. Glycosaminoglycan	87. Resistin
	49. Glycosidase	88. Stromelysin
	50. Growth factor	89. TIMP
	51. Hexosaminidase	90. Transferrin
	52. Hyaluronic	91. Urate
	53. Hydroxydeoxyguanosine	92. Visfatin
93. 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22 or 23 or 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32 or 33 or 34 or 35 or 36 or 37 or 38 or 39 or 40 or 41 or 42 or 43 or 44 or 45 or 46 or 47 or 48 or 49 or 50 or 51 or 52 or 53 or 54 or 55 or 56 or 57 or 58 or 59 or 60 or 61 or 62 or 63 or 64 or 65 or 66 or 67 or 68 or 69 or 70 or 71 or 72 or 73 or 74 or 75 or 76 or 77 or 78 or 79 or 80 or 81 or 82 or 83 or 84 or 85 or 86 or 87 or 88 or 89 or 90 or 91 or 92		
94. 3 AND 4 AND 93		

2.3.3.2. Selection of Studies Using a Dual Procedure: Data Mining and Manual Methods

The manipulation of the data identified in the searches was performed out using the R software (version 3.4.3) and packages downloaded from the Comprehensive R Archive Network [21].

A total of 176 searches based on the combination of the previously defined terms were performed in each database. Considering the principles of research reproducibility [22], the abstracts of all the articles were analysed computationally applying a series of positive and negative words. The automated data mining process was previously validated in a small group of diagnostic accuracy papers that met the inclusion criteria. The analysis of the positive and negative words was carried out using the tm package, version 0.7-5, and NLP package, version 0.1-11 [23,24].

Only papers with multiple published identifiers (PMIDs), those with a single PMID that did not present an abstract, or those that did not have a designated PMID were analysed manually.

The manual selection was conducted by two independent reviewers (NAB, ARI). Those articles with at least one positive word and no negative word were selected as candidates for their full text to be assessed. The dual procedure of selection of studies and the list of positive and negative words have been explained in detail in Objective 1.

2.3.4. Data Collection and Analysis

2.3.4.1. Selection of Studies

The analysis of the full texts was carried out by two independent reviewers (NAB, ARI), applying the protocol previously described in Objective 1. The reasons for study exclusion were recorded.

2.3.5. Data Extraction and Management

Four authors (NAB, ARI, IT and CC) independently extracted data using a standardised data collection form. On the one hand, the first two authors focused on the characteristics of the articles: type of accuracy study, characteristics of the patient groups, definition of the reference standard, number and type of control and target conditions at the patient-level, type of salivary sample, type of molecular biomarker analysed and the technique used for the detection of biomarkers. On the other hand, the second two authors analysed the accuracy data of the studies: contingency table based on true positives, true negatives, false positives, false negatives, sensitivity and specificity values, and classification thresholds.

2.3.6. Assessment of Methodological Quality

Two review authors (IT and CBC) independently analysed the quality of the included studies using the critical review checklist of the revised Quality Assessment of Diagnostic Studies (QUADAS-2) [25]. Considering the guidelines of Whiting et al. [25], this checklist was previously modified according to the premise of the "objective" quantification of salivary biomarkers, which allowed the elimination of a question from domain 2. The description and method of application of the modified QUADAS-2 checklist can be found in Objective 1.

2.3.7. Qualitative Analysis

The unit of analysis was each 2x2 contingency table of a biomarker in saliva. Biomarkers were grouped into four types: 1) enzymes; 2) inflammatory mediators; 3) tissue breakdown products; 4) others [11,26].

In most studies, the concentration or level of a quantifiable molecule was reported. Consequently, these results were interpreted as positive or negative based on a numerical measurement that was categorised according to a biomarker classification threshold (pre-stated or not). Since the unit of analysis is the contingency table, an article could present more than one table according to different control or target conditions and different techniques used for the quantification of the same biomarker. The diagnostic accuracy of a biomarker was evaluated by measures of its capacity to detect the presence or absence of a target condition, such as the presence of periodontitis.

When an article reported the same contingency table in terms of the index test and the clinical diagnosis using the standard reference for different thresholds, the one with the highest value of the Youden index was chosen [27].

Estimates of accuracy were expressed as sensitivity (SENS) and specificity (SPEC) values, and with 95% confidence intervals (CIs), for each classification of a biomarker in saliva. They were then displayed as coupled forest plots. These graphics were performed with the mada

package (version 0.5.8) [28]. Other performance measures, such as the positive predictive value (PPV), the negative predictive value (NPV), the positive likelihood ratio (LR+), the negative likelihood ratio (LR-) the diagnostic odds ratio (DOR) and the Youden's index, were also calculated using the data extracted from each paper.

2.3.8. Quantitative Analysis

A meta-analysis was performed when the number of contingency tables of a biomarker in saliva was at least three from at least three articles. The analysis strategy was to include all accuracy studies regardless of the threshold value. Hierarchical summary receiver operating characteristic (HSROC) modelling was used to conduct the meta-analysis. The calculation of the HSROC model was performed using the HSROC package, version 2.1.8 [29], whereby estimations are carried out using a Bayesian approach, implemented via a Gibbs sampler [29]. An explanation of the main features of HSROC modelling is given in Objective 1.

To attempt to provide direct evidence for the usefulness or effectiveness of the salivary biomarkers subjected to meta-analytical analysis [30], we showed the summary accuracy data obtained from meta-analyses using natural frequencies based on a hypothetical cohort of 1000 patients [31]. These calculations were made, taking into account summary estimates of sensitivity, specificity, and different prevalence of periodontitis.

2.3.8.1. Investigations of Heterogeneity

In the present review, the threshold effect was first evaluated graphically by observing the coupled forest plot. If there was a threshold effect, the sensitivity and specificity changed inversely, showing the coupled forest plot as a V or an inverted-V shape [32]. Second, the threshold effect was statistically evaluated by a linear correlation value between sensitivity and the false-positive rate (1-specificity) using the Spearman correlation coefficient. A threshold effect will be present if the correlation value obtained is 0.6 or higher [33].

Other analyses to explore other sources of heterogeneity, as well as sensitivity analysis, could not be performed, as none of the biomarkers presented a minimum of 10 classifications per possible covariable of interest.

2.4. Results

2.4.1. Study Selection

In total, after the automated removal of duplicates, 4511 articles were obtained from the six research databases. Then, 90.5% of the abstracts were evaluated using data-mining techniques and the remaining 9.5% with a manual procedure; a total of 104 full-text articles were assessed for eligibility. Also, six more were detected after analysing the references from a list of reviews and full-text papers.

In the eligibility phase, 92 articles were excluded for various reasons (Appendix S2), meaning that 18 publications (86 contingency tables) were evaluated in qualitative analysis. After applying the established requirements for meta-analyses, 12 articles (36 contingency tables) were considered for the quantitative analysis. A detailed flow chart is shown in Figure 1.

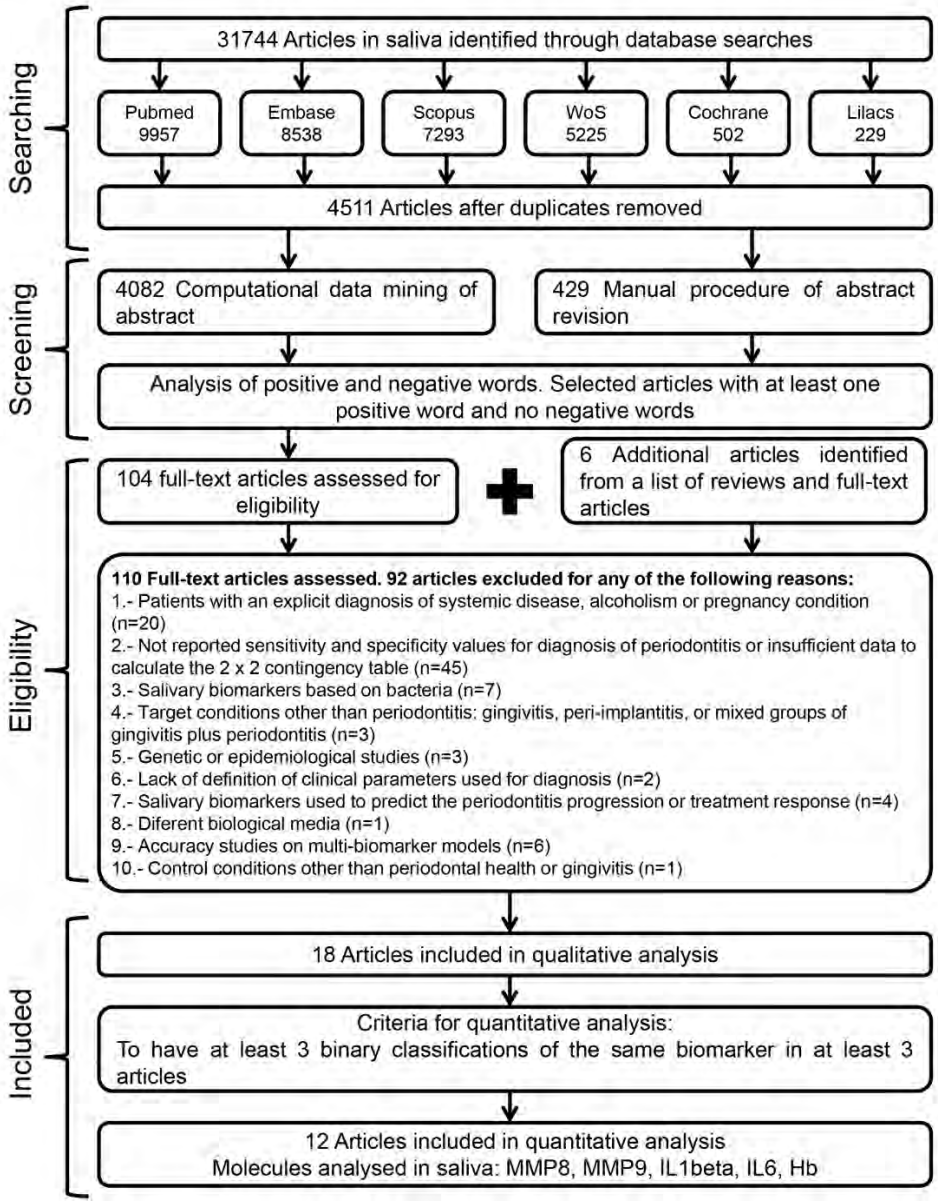


Figure 1. Flow diagram of the literature search and selection of the studies, adapted from the Cochrane protocol [19].

2.4.2. Characteristics of Diagnostic Accuracy Studies in Saliva

Table 2 contains a quantitative summary of the main descriptive characteristics of the included articles. Investigation of the diagnostic accuracy of only one biomarker in saliva was observed in 8/18 articles (44.4%), while in the remaining 10/18 (55.6%) more than one salivary biomarker was evaluated. A total of 32 individual molecular biomarkers were identified, of which 13 (40.6%) were inflammatory and host-response mediators, nine (28.1%) were enzymes, six (18.8%) were periodontal breakdown-related products and four (12.5%) were classified as "others".

The most common control condition was periodontal health status (65.1%), followed by a combined group of periodontal health and gingivitis (18.6%), with the remaining 16.3% consisting of undefined non-periodontal patients. The most commonly identified target condition was chronic periodontitis with different degrees of extent and severity (86.0%), followed by periodontitis (11.6%) and a combined group of chronic and aggressive periodontitis (2.4%). In most of the included studies, study groups consisted of non-smokers and smokers (11/18, 61.1%), while only non-smoking patients were assessed at 6/18 (33.3%).

Regarding the type of saliva sample analysed, there was a predominance of non-stimulated saliva (63.2% of the series), followed by stimulated saliva and rinsing (21.0% and 15.8%, respectively); other aspects such as the storage temperature of the samples, the most frequent is storage at -80ºC for further processing (in 50.0% of the series). The most commonly used techniques for the identification of salivary biomarkers were ELISA (54.4%) and multiparametric cytometry (17.6%).

Table 2. Biomarkers in saliva analysed, characteristics of controls and target conditions and technique applied for the detection of biomarkers; definition of the index tests included in the meta-analysis (n=18 articles).

	Index Test		Control Condition		Target Condition		Technique	
No.	Name (Family)	Number of Evaluation	Range of Number of Samples	Types	Range of Number of Samples	Types	Name	Meta-analysis
1	MMP8 (E)	16/7	≤30 (10/4) 31-70 (1/1) 71-120 (5/2)	H (13/4) H and G (2/2) Non-P (1/1)	≤30 (5/3) 31-70 (6/4) 71-120 (4/3) 121-200 (1/1)	CP (1/1) CP-Lo (2/1) CP-Lo-Ge (1/1) CP-Mi-M-S (6/2) CP-Ge (1/1) CP-Ge-M (1/1) CP-Ge-S (2/1) CP-Ge-M-S (1/1) P (1/1)	ELISA (6/4) Multiplex cytometry (2/2) IFMA (4/1) POCID optical (2/1) POCID visual (2/1)	Y
2	IL1beta (I)	6/6	≤30 (4/4) 31-70 (1/1) 71-120 (1/1)	H (3/3) H and G (2/2) Non-P (1/1)	≤30 (1/1) 31-70 (4/4) 71-120 (1/1)	CP (1/1) CP and AgP (1/1) CP-Mi-M-S (2/2) CP-Ge-S (1/1) P (1/1)	ELISA (3/3) Multiplex cytometry (3/3)	Y
3	MMP9 (E)	6/3	≤30 (1/1) 31-70 (1/1) 71-120 (4/1)	H (4/1) H and G (1/1) Non-P (1/1)	≤30 (2/2) 31-70 (2/2) 71-120 (1/1) 121-200 (1/1)	CP-Lo (2/1) CP-Ge (1/1) CP-Lo-Ge (1/1) CP-Mi-M-S (1/1) CP-Ge-S (1/1)	ELISA (6/3)	Y
4	ICTP (T)	5/2	31-70 (1/1) 71-120 (4/1)	H (4/1) H and G (1/1)	≤30 (1/1) 31-70 (2/2) 71-120 (1/1) 121-200 (1/1)	CP-Lo (2/1) CP-Ge (1/1) CP-Lo-Ge (1/1) CP-Mi-M-S (1/1)	ELISA (5/2)	N
5	IL6 (I)	4/4	≤30 (2/2) 31-70 (1/1) 71-120 (1/1)	H (1/1) H and G (2/2) Non-P (1/1)	≤30 (1/1) 31-70 (2/2) 71-120 (1/1)	CP (1/1) CP-Mi-M-S (1/1) CP-Ge-S (1/1) P (1/1)	ELISA (1/1) Multiplex cytometry (3/3)	Y

6	Hb (T)	4/3	≤30 (2/1) 31-70 (1/1) 121-200 (1/1)	H-CPI0 (2/1) Non-P (2/2)	≤30 (2/1) 31-70 (1/1) 121-200 (1/1)	CP-CPI3 (1/1) CP-CPI4 (1/1) P (2/2)	Perioscreen test (1/1) OC-Hemodia Auto S assay (1/1) Nescauto SalivaHemo Plus assay (2/1)	Y
7	Ctx (T)	4/1	71-120 (4/1)	H (4/1)	≤30 (1/1) 31-70 (1/1) 71-120 (1/1) 121-200 (1/1)	CP-Lo (2/1) CP-Ge (1/1) CP-Lo-Ge (1/1)	ELISA (4/1)	N
8	MMP13 (E)	4/1	71-120 (4/1)	H (4/1)	≤30 (1/1) 31-70 (1/1) 71-120 (1/1) 121-200 (1/1)	CP-Lo (2/1) CP-Ge (1/1) CP-Lo-Ge (1/1)	ELISA (4/1)	N
9	Ntx (T)	4/1	71-120 (4/1)	H (4/1)	≤30 (1/1) 31-70 (1/1) 71-120 (1/1) 121-200 (1/1)	CP-Lo (2/1) CP-Ge (1/1) CP-Lo-Ge (1/1)	Osteomark assay (4/1)	N
10	TRACP5b (E)	4/1	71-120 (4/1)	H (4/1)	≤30 (1/1) 31-70 (1/1) 71-120 (1/1) 121-200 (1/1)	CP-Lo (2/1) CP-Ge (1/1) CP-Lo-Ge (1/1)	Bonetrap assay (4/1)	N
11	LDH (E)	3/2	≤30 (2/1) 31-70 (1/1)	H-CPI0 (2/1) Non-P (1/1)	≤30 (2/1) 31-70 (1/1)	P (1/1) CP-CPI3 (1/1) CP-CPI4 (1/1)	L type Wako LDH J assay (3/2)	N
12	MIP1alpha (I)	2/2	31-70 (1/1) 71-120 (1/1)	H (1/1) H and G (1/1)	31-70 (1/1) 71-120 (1/1)	CP-Ge-M-S (1/1) P (1/1)	ELISA (1/1) Multiplex cytometry (1/1)	N
13	Nitric oxide (O)	2/2	≤30 (2/2)	H (1/1) H and G (1/1)	≤30 (2/2)	CP (1/1) P (1/1)	ELISA (1/1) CGR Spectrophotometry (1/1)	N
14	PGE2 (I)	2/2	≤30 (2/2)	H (2/2)	31-70 (2/2)	CP (1/1) CP-Mi-M-S (1/1)	ELISA (2/2)	N
15	TNFalpha (I)	2/2	≤30 (2/2)	H (1/1) Non-P (1/1)	≤30 (1/1) 31-70 (1/1)	CP (1/1) CP-Ge-S (1/1)	Multiplex cytometry (2/2)	N
16	Cysteine (E)	2/1	≤30 (2/1)	H (2/1)	≤30 (2/1)	CP (2/1)	HPLC (2/1)	N

17	Albumin (O)	1/1	≤30 (1/1)	H (1/1)	31-70 (1/1)	CP (1/1)	ELISA (1/1)	N
18	BG (E)	1/1	≥201 (1/1)	H (1/1)	31-70 (1/1)	CP (1/1)	Fluor assay (1/1)	N
19	Calprotectin (T)	1/1	31-70 (1/1)	H and G (1/1)	31-70 (1/1)	CP-Mi-M-S (1/1)	ELISA (1/1)	N
20	CRP (I)	1/1	≤30 (1/1)	Non-P (1/1)	≤30 (1/1)	CP-Ge-S (1/1)	ELISA (1/1)	N
21	IFNalpha (I)	1/1	≤30 (1/1)	H (1/1)	31-70 (1/1)	CP (1/1)	Multiplex cytometry (1/1)	N
22	IL8 (I)	1/1	≤30 (1/1)	Non-P (1/1)	≤30 (1/1)	CP-Ge-S (1/1)	Multiplex cytometry (1/1)	N
23	IL10 (I)	1/1	31-70 (1/1)	H and G (1/1)	31-70 (1/1)	CP-Mi-M-S (1/1)	ELISA (1/1)	N
24	IL1ra (I)	1/1	≤30 (1/1)	Non-P (1/1)	≤30 (1/1)	CP-Ge-S (1/1)	Multiplex cytometry (1/1)	N
25	Lactoferrin (I)	1/1	≤30 (1/1)	Non-P (1/1)	≤30 (1/1)	CP-Ge-S (1/1)	ELISA (1/1)	N
26	MPO (E)	1/1	31-70 (1/1)	H and G (1/1)	≤30 (1/1)	P (1/1)	Colour assay (1/1)	N
27	Nitrate (O)	1/1	≤30 (1/1)	H and G (1/1)	≤30 (1/1)	P (1/1)	ELISA (1/1)	N
28	Nitrite (O)	1/1	≤30 (1/1)	H and G (1/1)	≤30 (1/1)	P (1/1)	ELISA (1/1)	N
29	OPG (T)	1/1	31-70 (1/1)	H and G (1/1)	31-70 (1/1)	CP-Mi-M-S (1/1)	ELISA (1/1)	N
30	PDGF (I)	1/1	≤30 (1/1)	Non-P (1/1)	≤30 (1/1)	CP-Ge-S (1/1)	Multiplex cytometry (1/1)	N
31	Trappin2 (E)	1/1	≤30 (1/1)	H (1/1)	31-70 (1/1)	CP and AgP (1/1)	ELISA (1/1)	N
32	VEGF (I)	1/1	≤30 (1/1)	Non-P (1/1)	≤30 (1/1)	CP-Ge-S (1/1)	Multiplex cytometry (1/1)	N

2.4.3. Quality Assessment of Diagnostic Accuracy Studies in Saliva

The results on the quality of the diagnostic studies obtained using the QUADAS-2 tool in the different domains are shown in Figure 2. In the patient selection domain, 15/18 (83.3%) and 14/18 of papers (77.8%) had a cross-sectional and case-control design, respectively, while 14/18 of articles (77.8%) were catalogued as having a high risk of bias. In the index text domain, the question about the application of a pre-specified threshold was judged to be "no" in 16/18 articles (88.9%), indicating a high risk of bias.

The reference standard correctly classified the periodontal status without knowledge of the results of the index test in 16/18 (88.9%) so in these articles, the standard reference domain was related to low risk of bias. However, it is important to consider that in only seven studies (38.9%), the authors confirmed the practice of calibration methods or the involvement of calibrated professionals for the recording of clinical status. All participants in all included studies received the same reference standard, with 61.1% of them (11/18 articles) having an appropriate interval between the clinical reference and the index test; in 9/18 of articles (50.0%), this domain was defined as being of low risk of bias.

Concerns about the applicability of all the domains for all the included articles received a “low” judgement, except two papers for the standard reference domain (they were considered to be "unclear", as the authors applied the CPI system as a standard reference).

Considering the sample size as a parameter of methodological quality, series of ≤30 patients with the control condition prevailed in 46.5% of the contingency tables; between 31 and 70 control patients were included in 14%; and >71 patients in 39.5%. Regarding the number of subjects with the target condition, the study group was composed of: ≤30 patients in 37.2% of the contingency tables, between 31 and 70 patients in 38.4%; and >71 patients in 24.4% (Table 2).

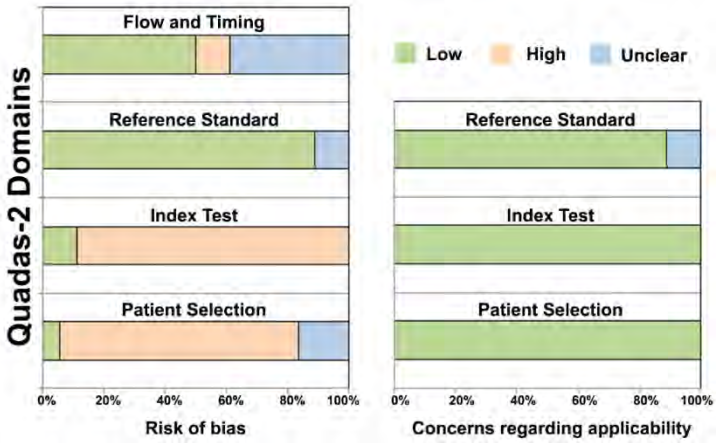


Figure 2. Quality assessment according to the QUADAS-2 tool: risk of bias and applicability concerns.

2.4.4. Synthesis of the Qualitative and Quantitative Analyses of Five Salivary Biomarkers

Of the 32 biomarkers, only five had at least three contingency tables in at least three articles, and it was on these that the meta-analyses were performed. In terms of the type of biomarker, these molecules were: enzymes (matrix metalloproteinase -MMP- 8, 16 contingency tables/seven articles; MMP9, six/three) [34-40]; inflammatory and host-response mediators (interleukin -IL- 1beta, six/six; IL6, four/four) [36-38,40-42]; and tissue breakdown products (haemoglobin -Hb-, four/three) [43-45].

The ACC range (sensitivity and specificity ranges) were broad for five biomarkers and these were: 87.8-62.4% (93.3-55.6% and 86.7-48.1%) for MMP8; 85.5-57.6% (93.1-33.3% and 82.7-48.1%) for MMP9; 90.0-54.4% (88.0-53.8% and 96.8-51.9%) for IL1beta; 91.3-50.9% (88.0-53.3% and 96.7-48.1%) for IL6; and 76.1-62.2% (75.9-66.7% and 78.6-60.7%) for Hb. The CIs of the five biomarkers were wide, with those of MMP8 having a narrower range (95% CIs for sensitivity= 54.6-91.9%; 95% CIs for specificity= 50.7-91.2%). None of these biomarkers showed a threshold effect, either graphically or statistically (Figures 3A, 3B, 3C and Table 3).

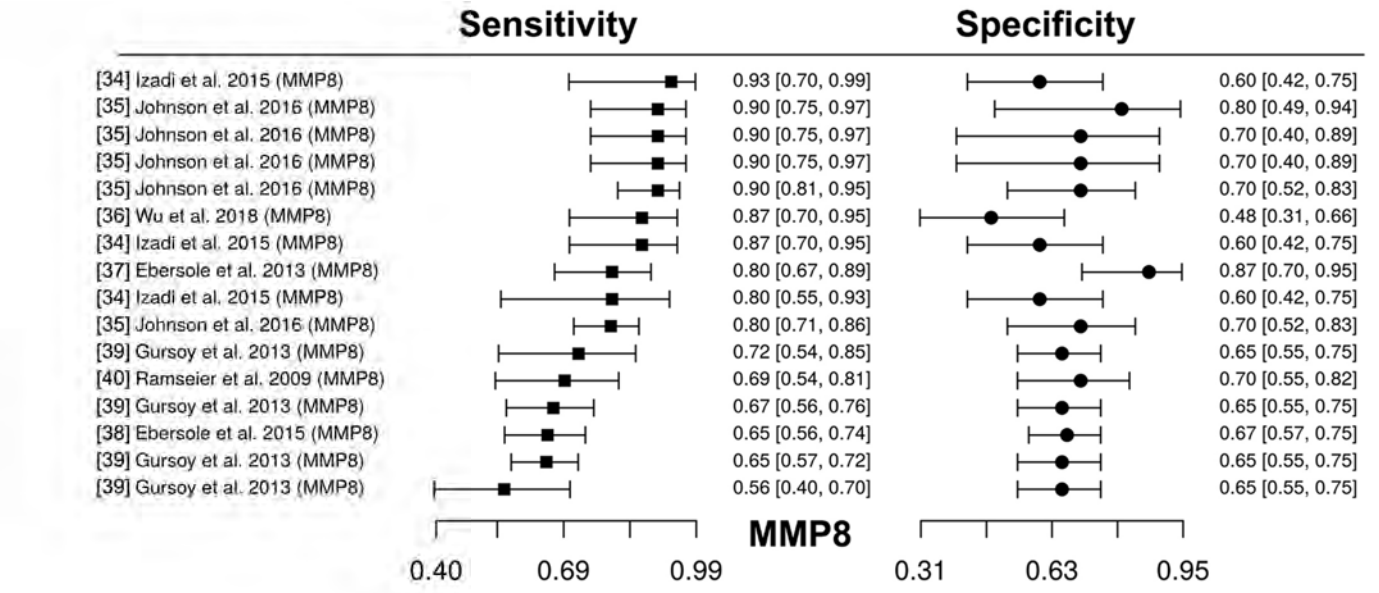


Figure 3A**.** Coupled forest plot with diagnostic test accuracy (sensitivity, specificity, and 95% confidence interval) of the salivary MMP8 classification included, presented at least three classifications from at least three articles. In the coupled forest plot, sensitivity and specificity values are rounded, and the biomarkers are listed according to the sensitivity value (from highest to lowest value).

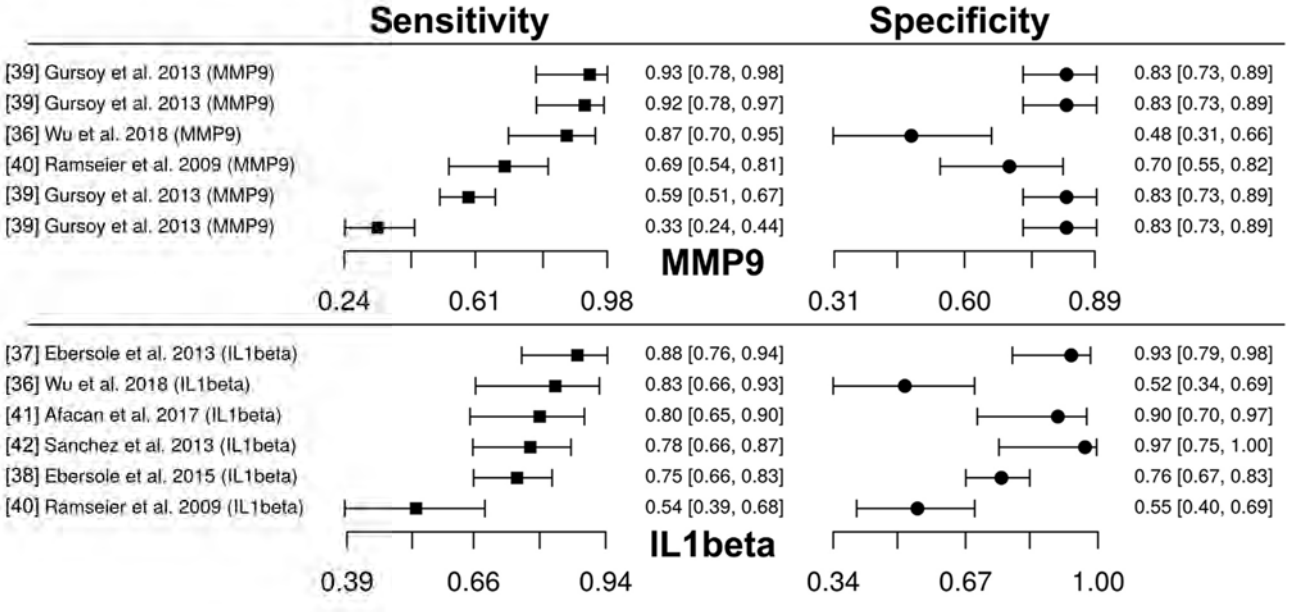


Figure 3B. Coupled forest plot with diagnostic test accuracy (sensitivity, specificity, and 95% confidence interval) of salivary MMP9 and IL1beta classifications included, presented at least three classifications from at least three articles.In the coupled forest plot, sensitivity and specificity values are rounded, and the biomarkers are listed according to the sensitivity value (from highest to lowest value).

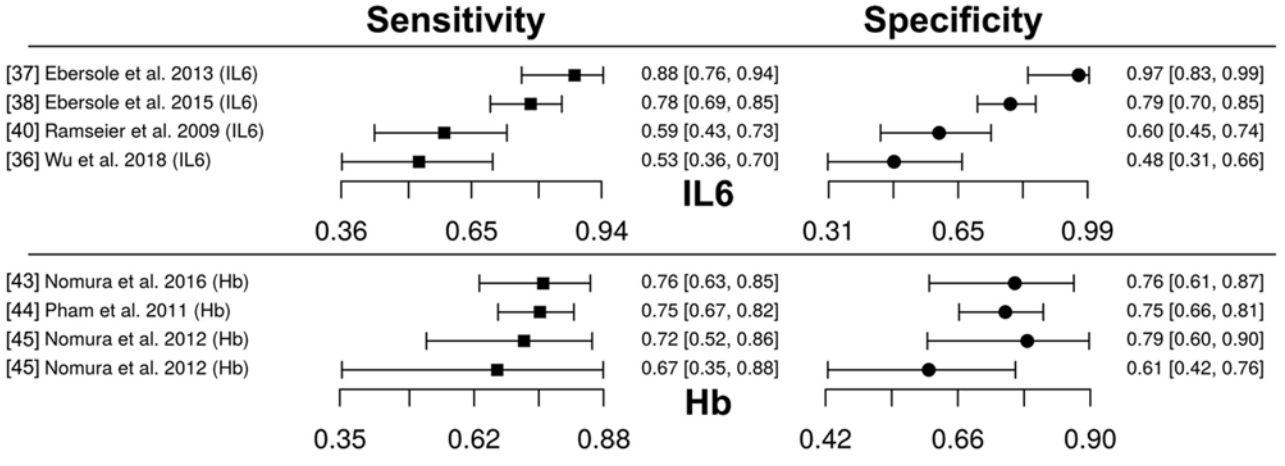


Figure 3C. Coupled forest plot with diagnostic test accuracy (sensitivity, specificity, and 95% confidence interval) of salivary IL6 and Hb classifications included, presented at least three classifications from at least three articles. In the coupled forest plot, sensitivity and specificity values are rounded, and the biomarkers are listed according to the sensitivity value (from highest to lowest value).

Table 3. Diagnostic test accuracy measurements for each salivary biomarker classification included, presented at least three classifications from at least three articles.

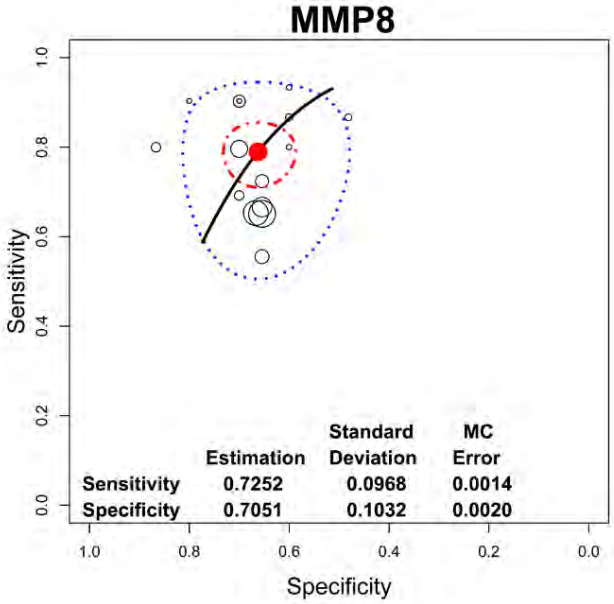
Study (first author)	Index Test	No. CC/ No. TC	Threshold (units)	AUC	ACC (%)	SENS/ SPEC (%)	PPV/ NPV (%)	LR+/ LR-	DOR	J Index
Izadi [34]	MMP8	30/15	25.0 (ng/ml)	NSp	71.1	93.3/ 60.0	53.8/ 94.7	2.3/ 0.1	21.0	0.53
Johnson [35]		10/31	8.0 (ng/ml)	0.860	87.8	90.3/ 80.0	93.3/ 72.7	4.5/ 0.1	37.3	0.70
Johnson [35]		30/72	NSp (optical units)	NSp	85.4	90.3/ 70.0	90.3/ 70.0	3.0/ 0.1	21.8	0.60
Johnson [35]		10/31	NSp (score)	0.780	85.4	90.3/ 70.0	90.3/ 70.0	3.0/ 0.1	21.8	0.60
Johnson [35]		10/31	NSp (score)	0.830	84.3	90.3/ 70.0	87.8/ 75.0	3.0/ 0.1	21.7	0.60
Wu [36]		27/30	36733.1 (ng/ml)	NSp	68.4	86.7/ 48.1	65.0/ 76.5	1.7/ 0.3	6.0	0.35
Izadi [34]		30/30	25.0 (ng/ml)	NSp	73.3	86.7/ 60.0	68.4/ 81.8	2.2/ 0.2	9.8	0.47
Ebersole [37]		30/50	110.0 (ng/ml)	0.920	82.5	80.0/ 86.7	90.9/ 72.2	6.0/ 0.2	26.0	0.67
Izadi [34]		30/15	25.0 (ng/ml)	NSp	66.7	80.0/ 60.0	50.0/ 85.7	2.0/ 0.3	6.0	0.40
Johnson [35]		30/113	NSp (optical units)	0.680	77.6	79.6/ 70.0	90.9/ 47.7	2.7/ 0.3	9.1	0.50
Gursoy [39]		81/29	383.9 (ng/ml)	NSp	67.3	72.4/ 65.4	42.9/ 86.9	2.1/ 0.4	5.0	0.38
Ramseier [40]		40/39	87.0 (ng/ml)	0.750	69.6	69.2/ 70.0	69.2/ 70.0	2.3/ 0.4	5.3	0.39
Gursoy [39]		81/84	383.9 (ng/ml)	NSp	66.1	66.7/ 65.4	66.7/ 65.4	1.9/ 0.5	3.8	0.32
Ebersole [37]		108/101	165.9 (ng/ml)	0.728	66.0	65.3/ 66.7	64.7/ 67.3	2.0/ 0.5	3.8	0.32
Gursoy [39]		81/36	383.9 (ng/ml)	NSp	65.2	65.1/ 65.4	77.6/ 50.5	1.9/ 0.5	3.5	0.31

Gursoy [39]		81/149	383.9 (ng/ml)	NSp	62.4	55.6/ 65.4	41.7/ 76.8	1.6/ 0.7	2.4	0.21
Gursoy [39]	MMP9	81/29	126.9 (ng/ml)	NSp	85.5	93.1/ 82.7	65.9/ 97.1	5.4/ 0.1	64.6	0.76
Gursoy [39]		81/36	126.9 (ng/ml)	NSp	85.5	91.7/ 82.7	70.2/ 95.7	5.3/ 0.1	52.6	0.74
Wu [36]		27/30	65000.0 (ng/ml)	NSp	68.4	86.7/ 48.1	65.0/ 76.5	1.7/ 0.3	6.0	0.35
Ramseier [40]		40/39	240.0 (ng/ml)	0.720	69.6	69.2/ 70.0	69.2/ 70.0	2.3/ 0.4	5.3	0.39
Gursoy [39]		81/149	126.9 (ng/ml)	NSp	67.4	59.1/ 82.7	86.3/ 52.3	3.4/ 0.5	6.9	0.42
Gursoy [39]		81/84	126.9 (ng/ml)	NSp	57.6	33.3/ 82.7	66.7/ 54.5	1.9/ 0.8	2.4	0.16
Ebersole [37]	IL1beta	30/50	17.8 (pg/ml)	0.950	90.0	88.0/ 93.3	95.7/ 82.4	13.2/ 0.1	102.7	0.81
Wu [36]		27/30	71.5 (pg/ml)	NSp	68.4	83.3/ 51.9	65.8/ 73.7	1.7/ 0.3	5.4	0.35
Afacan [41]		20/40	445.7 (pg/mg)	0.950	83.3	80.0/ 90.0	94.1/ 69.2	8.0/ 0.2	36.0	0.70
Sánchez [42]		15/59	212.0 (pg/ml)	0.960	81.9	78.0/ 96.8	98.9/ 53.6	24.2/ 0.2	106.2	0.75
Ebersole [38]		108/101	24.0 (pg/ml)	0.830	75.6	75.2/ 75.9	74.5/ 76.6	3.1/ 0.3	9.6	0.51
Ramseier [40]		40/39	235.8 (pg/ml)	0.720	54.4	53.8/ 55.0	53.8/ 55.0	1.2/ 0.8	1.4	0.09
Ebersole [37]	IL6	30/50	7.5 (pg/ml)	0.950	91.3	88.0/ 96.7	97.8/ 82.9	26.4/ 0.1	212.7	0.85
Ebersole [38]		108/ 101	5.1 (pg/ml)	.0849	78.5	78.2/ 78.7	77.5/ 79.4	3.7/ 0.3	13.3	0.57
Ramseier [40]		40/39	22.4 (pg/ml)	0.710	59.5	59.0/ 60.0	59.0/ 60.0	1.5/ 0.7	2.2	0.19
Wu [36]		27/30	3.7 (pg/ml)	NSp	50.9	53.3/ 48.1	53.3/ 48.1	1.0/ 1.0	1.1	0.02

Nomura [43]	Hb	38/54	1.2 (µg/ml)	0.846	76.1	75.9/ 76.3	82.0/ 69.0	3.2/ 0.3	10.2	0.52
Pham [44]		122/121	2.0 (score)	NSp	74.9	75.2/ 74.6	74.6/ 75.2	3.0/ 0.3	8.9	0.50
Nomura [45]		28/25	3.1 (µg/ml)	0.755	75.5	72.0/ 78.6	75.0/ 75.9	3.4/ 0.4	9.4	0.51
Nomura [45]		28/9	3.5 (µg/ml)	0.640	62.2	66.7/ 60.7	35.3/ 85.0	1.7/ 0.5	3.1	0.27

Biomarkers are listed according to the sensitivity value (from highest to lowest value).

Figures 4A, 4B and 4C show the meta-analyses performed on the five biomarkers in saliva using HSROC modelling. In terms of the sensitivity estimations ± standard deviation, the best values were obtained by IL1beta (78.7 ± 19.9%), followed by MMP8 (72.5 ± 9.6%), IL6 and Hb (72.0 % for both molecules ± 23.2% and 13.6%, respectively); the worst sensitivity values were for MMP9 (70.3 ± 23.3%). Regarding the results of the specificity estimations, MMP9 had the best values (81.5 ± 21.4%), followed by IL1beta (78.0 ± 19.9%) and Hb (75.2 ± 13.2%); MMP8 was associated with the lowest values (70.5 ± 10.3%). All four biomarkers showed an MC error value of sensitivity and specificity parameters smaller than 10% of its respective standard deviation, which means a high precision in the estimation of the parameters (Figures 4A, 4B, 4C and Appendix S3). Applying the premise of having at least three contingency tables of at least three articles, we performed various meta-analytical analyses of the five salivary biomarkers according to various selection criteria, the results of which are shown in Appendices S4-S8.



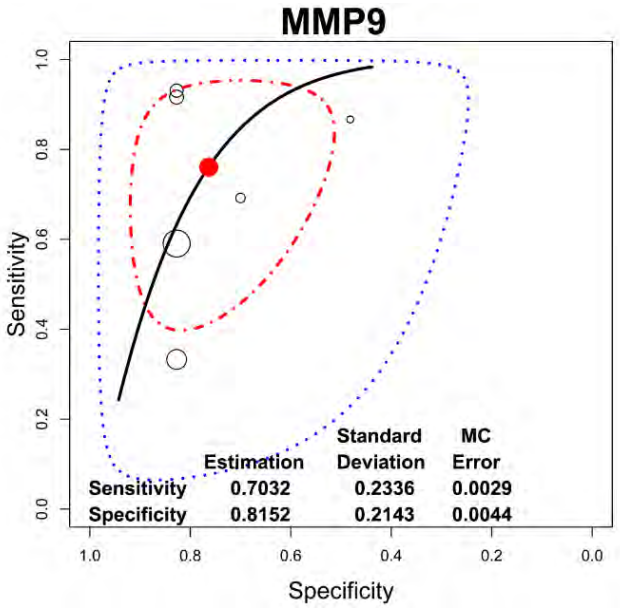
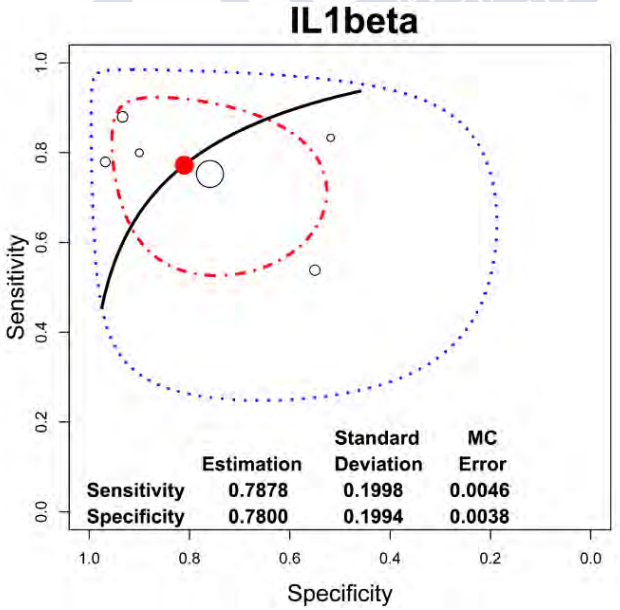


Figure 4A. Meta-analyses performed on MMP8 and MMP9 in saliva using HSROC modelling.



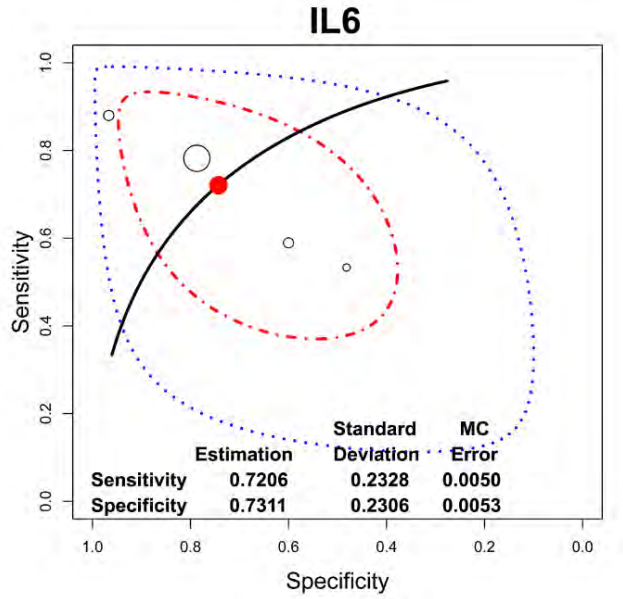


Figure 4B. Meta-analyses performed on IL1beta and IL6 in saliva using HSROC modelling.

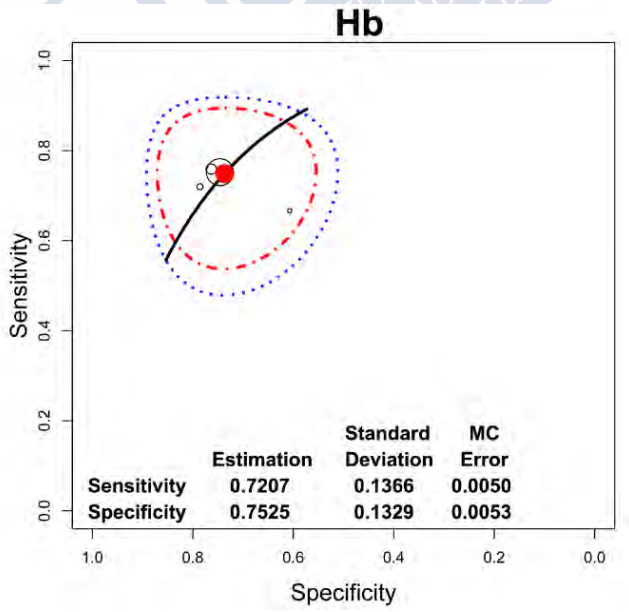


Figure 4C. Meta-analyses performed on Hb in saliva using HSROC modelling.

In relation to usefulness or effectiveness of the two most studied biomarkers in saliva (MMP8 and IL1beta), considering the accuracy estimators obtained from meta-analyses and a 45% prevalence of periodontitis [46,47], 67.2% of the total IL1beta positive tests would indicate a true positive; while of the total IL1beta negative tests, 78.7% would show a true negative. For a MMP8 test, these percentages would be 63.4% and 75.2%, respectively (Figures 5A and 5B).

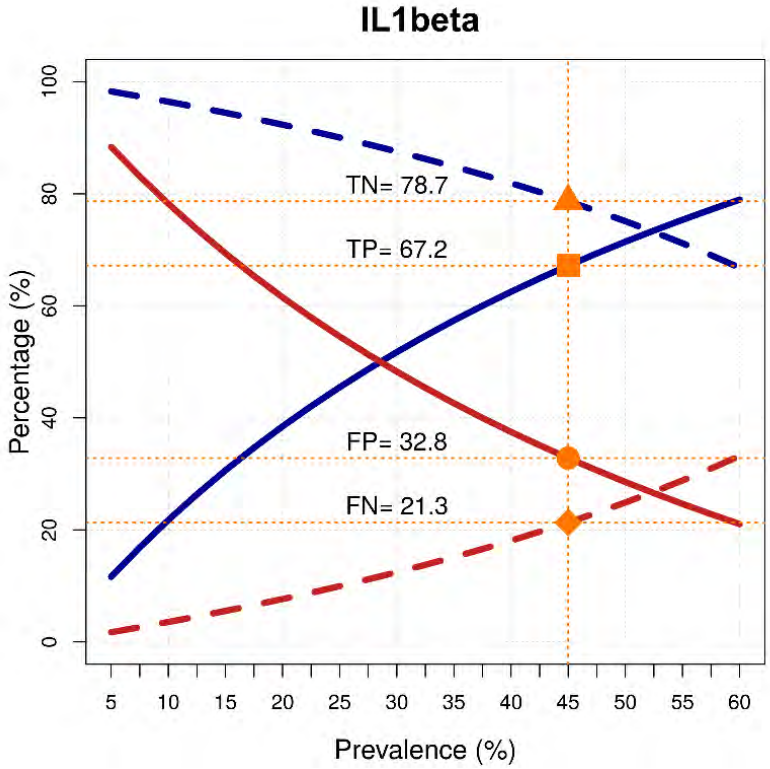


Figure 5A. Expression of summary accuracy data derived from meta-analyses using natural frequencies based on a hypothetical cohort of 1000 patients along different prevalences of periodontitis for the IL1beta biomarker.

Discontinuous lines indicate the percentage of negative tests for the different prevalences of periodontitis (blue line, the percentage of true negatives and red line, the percentage of false negatives). The continuous lines indicate the percentage of positive tests for the different prevalences of periodontitis (blue line, the percentage of true positives and the red line, the percentage of false positives).

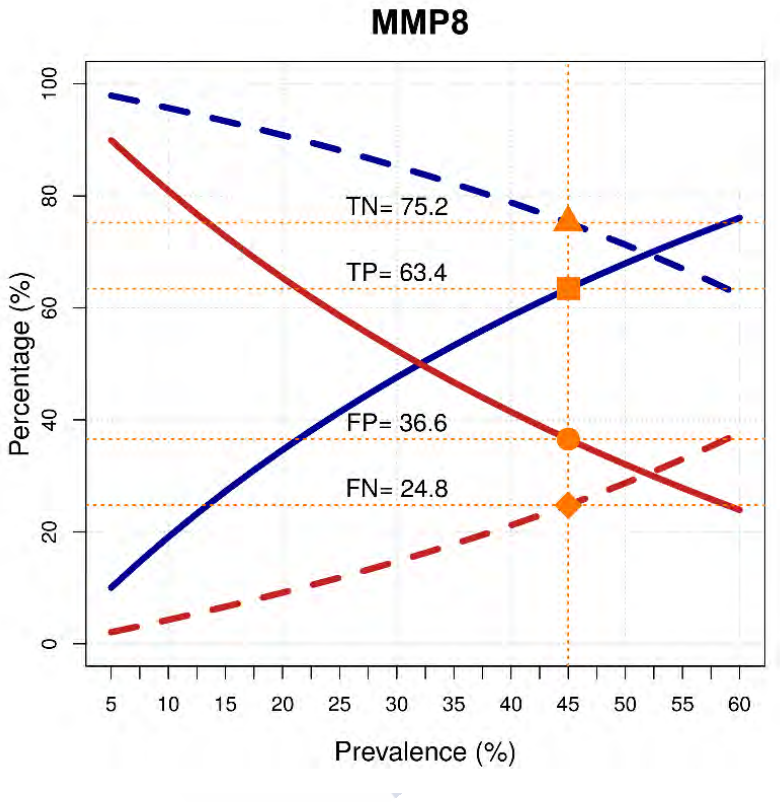


Figure 5B. Expression of summary accuracy data derived from meta-analyses using natural frequencies based on a hypothetical cohort of 1000 patients along different prevalences of periodontitis for the MMP8 biomarker.

Discontinuous lines indicate the percentage of negative tests for the different prevalences of periodontitis (blue line, the percentage of true negatives and red line, the percentage of false negatives). The continuous lines indicate the percentage of positive tests for the different prevalences of periodontitis (blue line, the percentage of true positives and the red line, the percentage of false positives).

2.4.5. Results of the Qualitative Analysis of the Remaining Biomarkers

Figures 6A, 6B, 6C and 6D set out the forest plots of the 27 remaining biomarkers, while Table 4 contains all the diagnostic classification parameters. If we focus on those salivary biomarkers that showed better ACC values, above 90%, for diagnosis of periodontitis (sensitivity/specificity values), Tassi and Lotito [48] obtained an ACC of 97.1% (97.3/96.8%) for cysteine, Al-Sabbagh et al. [49] an ACC of 93.8% (95.0/92.5%) for MIP1alpha, and Bejer-Mir et al. [50] an ACC of 95.2% (92.9/96.4%) for nitric oxide, nitrate and nitrite.

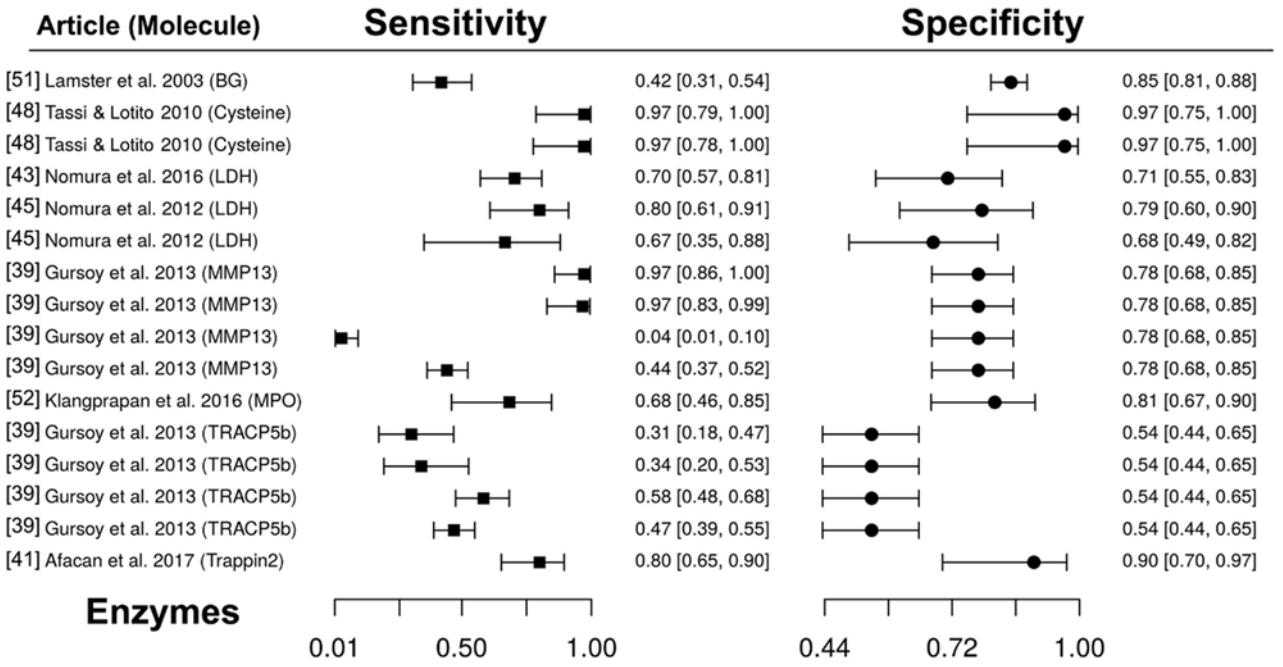


Figure 6A**.** Coupled forest plot with diagnostic test accuracy (sensitivity, specificity, and 95% confidence interval) of salivary enzymes classifications included, which was presented in less than three articles. In the coupled forest plot, sensitivity and specificity values are rounded, and the biomarkers were listed in alphabetical order within each type of biomarker.

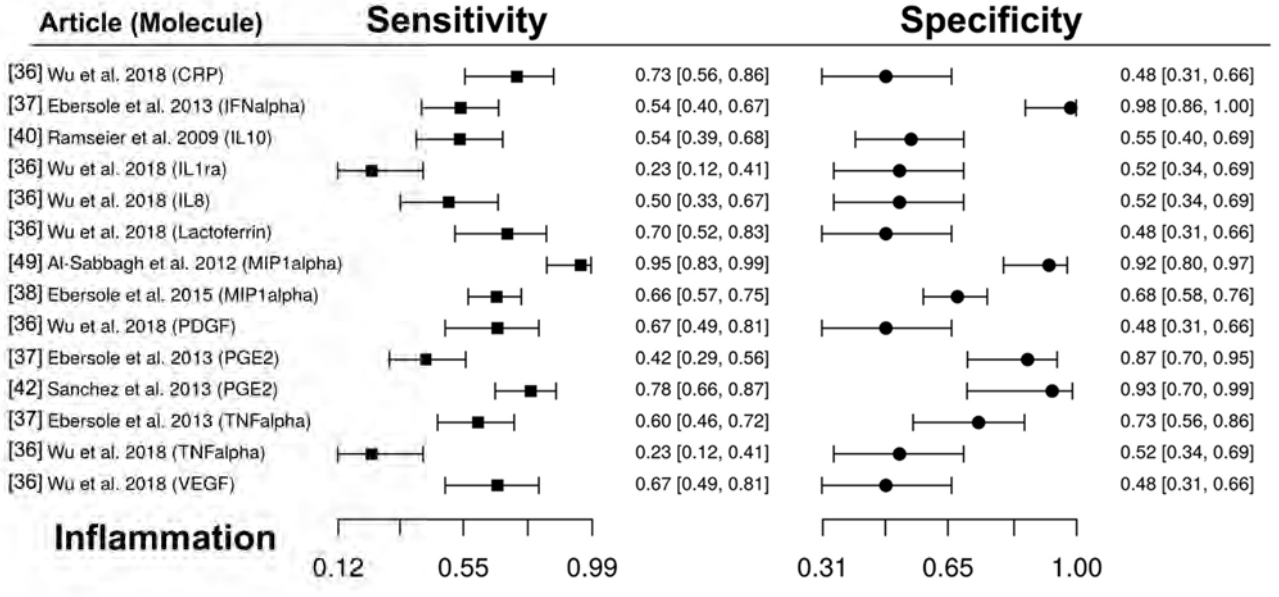


Figure 6B. Coupled forest plot with diagnostic test accuracy (sensitivity, specificity, and 95% confidence interval) of the classifications of the inflammation molecules included, which was presented in less than three articles. In the coupled forest plot, sensitivity and specificity values are rounded, and the biomarkers were listed in alphabetical order within each type of biomarker.

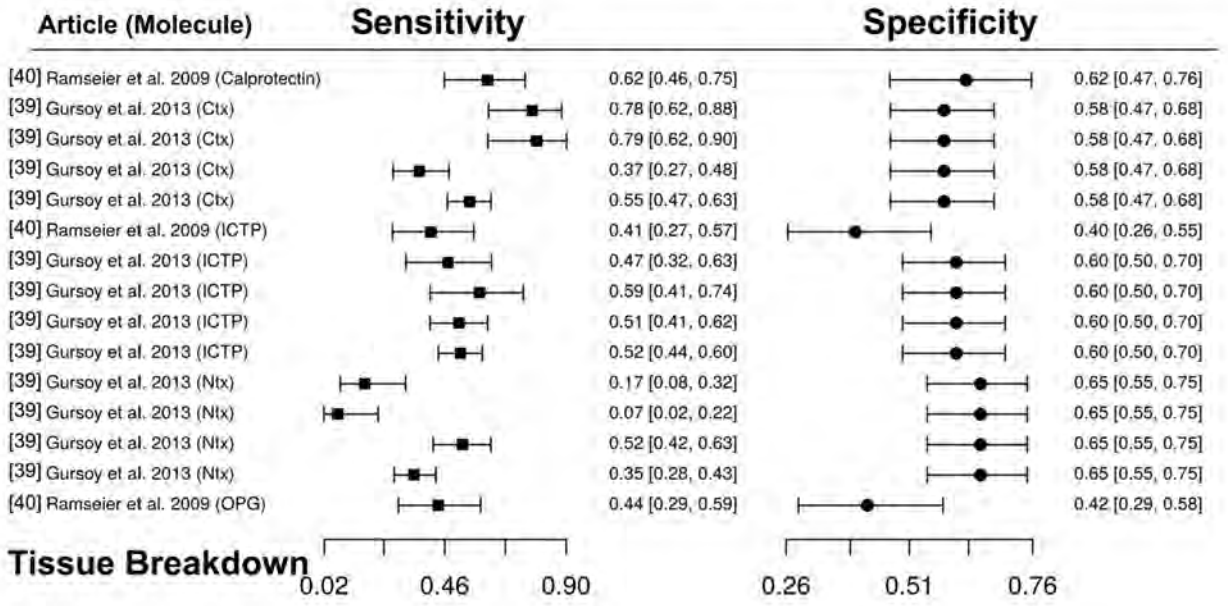


Figure 6C. Coupled forest plot with diagnostic test accuracy (sensitivity, specificity, and 95% confidence interval) of the classifications of the tissue breakdown products included, which was presented in less than three articles. In the coupled forest plot, sensitivity and specificity values are rounded, and the biomarkers were listed in alphabetical order within each type of biomarker.

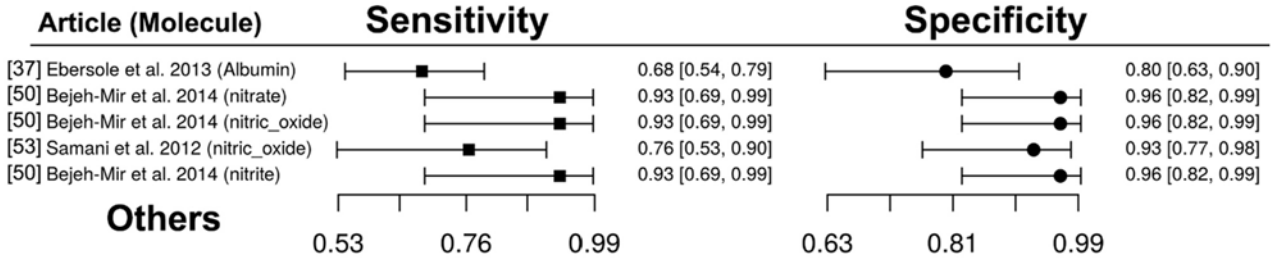


Figure 6D. Coupled forest plot with diagnostic test accuracy (sensitivity, specificity, and 95% confidence interval) of the classifications of other molecules included, which was presented in less than three articles. In the coupled forest plot, sensitivity and specificity values are rounded, and the biomarkers were listed in alphabetical order within each type of biomarker.

Table 4. Diagnostic test accuracy measurements for each biomarker classification included, which was presented in less than three articles. Biomarkers were listed in alphabetical order within each type of biomarker.

Study (first author)	Molecule	Type	No. CC/ No. TC	Threshold (units)	AUC	ACC (%)	SENS/ SPEC (%)	PPV/ NPV (%)	LR+/ LR-	DOR	J Index
Lamster [51]	BG	ENZYMES	312/69	100.0 (U)	NSp	77.2	42.0/ 84.9	38.2/ 86.9	2.8/ 0.7	4.1	0.27
Tassi [48]	Cysteine		15/18	1.85 (µMol/l)	1.000	97.1	97.3/ 96.8	97.3/ 96.8	30.2/ 0.0	1080.0	0.94
Tassi [48]	Cysteine		15/17	1.85 (µMol/l)	1.000	97.0	97.1/ 96.8	97.1/ 96.8	30.1/ 0.0	1020.0	0.94
Nomura [43]	LDH		38/54	298.0 (IU/l)	0.737	70.7	70.4/ 71.1	77.6/ 62.8	2.4/ 0.4	5.8	0.41
Nomura [45]	LDH		28/25	208.6 (NSp)	0.807	79.2	80.0/ 78.6	76.9/ 81.5	3.7/ 0.3	14.7	0.59
Nomura [45]	LDH		28/9	229.7 (NSp)	0.713	67.6	66.7/ 67.9	40.0/ 86.4	2.1/ 0.5	4.2	0.35

		ENZYMES									
Gursoy [39]	MMP13	81/36	0.16 (ng/ml)	NSp	83.8	97.2/ 77.8	66.0/ 98.4	4.4/ 0.0	122.5	0.75
Gursoy [39]	MMP13	81/29	0.16 (ng/ml)	NSp	82.7	96.6/ 77.8	60.9/ 98.4	4.3/ 0.0	98.0	0.74
Gursoy [39]	MMP13	81/84	0.16 (ng/ml)	NSp	40.0	3.6/ 77.8	14.3/ 43.8	0.2/ 1.2	0.1	-0.19
Gursoy [39]	MMP13	81/149	0.16 (ng/ml)	NSp	56.1	44.3/ 77.8	78.6/ 43.2	2.0/ 0.7	2.8	0.22
Klangprapan [52]	MPO	43/19	0.37 (465 nm)	0.749	77.4	68.4/ 81.4	61.9/ 85.4	3.7/ 0.4	9.5	0.50
Gursoy [39]	TRACP5b	81/36	0.37 (U/l)	NSp	47.0	30.6/ 54.3	22.9/ 63.8	0.7/ 1.3	0.5	-0.15
Gursoy [39]	TRACP5b	81/29	0.37 (U/l)	NSp	49.1	34.5/ 54.3	21.3/ 69.8	0.8/ 1.2	0.6	-0.11
Gursoy [39]	TRACP5b	81/84	0.37 (U/l)	NSp	56.4	58.3/ 54.3	57.0/ 55.7	1.3/ 0.8	1.7	0.13
Gursoy [39]	TRACP5b	81/149	0.37 (U/l)	NSp	49.6	47.0/ 54.3	65.4/ 35.8	1.0/ 1.0	1.1	0.01
Afacan [41]	Trappin2	20/40	45.52 (ng/mg)	0.910	83.3	80.0/ 90.0	94.1/ 69.2	8.0/ 0.2	36.0	0.70

Wu [36]	CRP	INFLAMMATION	27/30	1417.5 (pg/ml)	NSp	61.4	73.3/ 48.1	61.1/ 61.9	1.4/ 0.6	2.6	0.22
Ebersole [37]	IFNalpha		30/50	NSp (pg/ml)	0.750	70.8	54.0/ 98.4	98.2/ 56.6	32.9/ 0.5	70.4	0.52
Ramseier [40]	IL10		40/39	520.9 (pg/ml)	0.680	54.4	53.8/ 55.0	53.8/ 55.0	1.2/ 0.8	1.4	0.09
Wu [36]	IL1ra		27/30	8046.1 (pg/ml)	NSp	36.8	23.3/ 51.9	35.0/ 37.8	0.5/ 1.5	0.3	-0.25
Wu [36]	IL8		27/30	478.0 (pg/ml)	NSp	50.9	50.0/ 51.9	53.6/ 48.3	1.0/ 1.0	1.1	0.02
Wu [36]	Lactoferrin		27/30	10725.0 (ng/ml)	NSp	59.6	70.0/ 48.1	60.0/ 59.1	1.4/ 0.6	2.2	0.18
Al-Sabbagh [49]	MIP1alpha		40/40	1.12 (pg/ml)	0.940	93.8	95.0/ 92.5	92.7/ 94.9	12.7/ 0.1	234.3	0.88
Ebersole [38]	MIP1alpha		108/101	3.28 (pg/ml)	0.723	67.0	66.3/ 67.6	65.7/ 68.2	2.0/ 0.5	4.1	0.34
Wu [36]	PDGF		27/30	2.70 (ng/ml)	NSp	57.9	66.7/ 48.1	58.8/ 56.5	1.3/ 0.7	1.9	0.15
Ebersole [37]	PGE2		30/50	NSp (ng/ml)	0.490	58.8	42.0/ 86.7	84.0/ 47.3	3.2/ 0.7	4.7	0.29
Sánchez [42]	PGE2		15/59	121.40 (pg/ml)	0.920	81.1	78.0/ 93.3	97.9/ 51.9	11.7/ 0.2	49.5	0.71
Ebersole [37]	TNFalpha		30/50	NSp (pg/ml)	0.630	65.0	60.0/ 73.3	78.9/ 52.4	2.3/ 0.5	4.1	0.33
Wu [36]	TNFalpha		27/30	8.10 (pg/ml)	NSp	36.8	23.3/ 51.9	35.0/ 37.8	0.5/ 1.5	0.3	-0.25
Wu [36]	VEGF		27/30	721.60 (pg/ml)	NSp	57.9	66.7/ 48.1	58.8/ 56.5	1.3/ 0.7	1.9	0.15

Ramseier [40]	Calprotectin	TISSUE	40/39	3.60 (ng/ml)	0.680	62.0	61.5/ 62.5	61.5/ 62.5	1.6/ 0.6	2.7	0.24
Gursoy [39]	Ctx		81/36	0.28 (ng/ml)	NSp	64.1	77.8/ 58.0	45.2/ 85.5	1.9/ 0.4	4.8	0.36
Gursoy [39]	Ctx		81/29	0.28 (ng/ml)	NSp	63.6	79.3/ 58.0	40.4/ 88.7	1.9/ 0.4	5.3	0.37
Gursoy [39]	Ctx		81/84	0.28 (ng/ml)	NSp	47.3	36.9/ 58.0	47.7/ 47.0	0.9/ 1.1	0.8	-0.05
Gursoy [39]	Ctx		81/149	0.28 (ng/ml)	NSp	56.1	55.0/ 58.0	70.7/ 41.2	1.3/ 0.8	1.7	0.13
Ramseier [40]	ICTP		40/39	0.70 (ng/ml)	0.580	40.5	41.0/ 40.0	40.0/ 41.0	0.7/ 1.5	0.5	-0.19
Gursoy [39]	ICTP		81/36	7.27 (µg/ml)	NSp	56.4	47.2/ 60.5	34.7/ 72.1	1.2/ 0.9	1.4	0.08
Gursoy [39]	ICTP		81/29	7.27 (µg/ml)	NSp	60.0	58.6/ 60.5	34.7/ 80.3	1.5/ 0.7	2.2	0.19
Gursoy [39]	ICTP		81/84	7.27 (µg/ml)	NSp	55.8	51.2/ 60.5	57.3/ 54.4	1.3/ 0.8	1.6	0.12
Gursoy [39]	ICTP		81/149	7.27 (µg/ml)	NSp	54.8	51.7/ 60.5	70.6/ 40.5	1.3/ 0.8	1.6	0.12
Gursoy [39]	Ntx		81/36	0.07 (nM BCE)	NSp	50.4	16.7/ 65.4	17.6/ 63.9	0.5/ 1.3	0.4	-0.18
Gursoy [39]	Ntx		81/29	0.07 (nM BCE)	NSp	50.0	6.9/ 65.4	6.7/ 66.3	0.2/ 1.4	0.1	-0.28
Gursoy [39]	Ntx		81/84	0.07 (nM BCE)	NSp	58.8	52.4/ 65.4	61.1/ 57.0	1.5/ 0.7	2.1	0.18
Gursoy [39]	Ntx		81/149	0.07 (nM BCE)	NSp	45.7	34.9/ 65.4	65.0/ 35.3	1.0/ 1.0	1.0	0.00
Ramseier [40]	OPG		40/39	2.00 (pg/ml)	0.620	43.0	43.6/ 42.5	42.5/ 43.6	0.8/ 1.3	0.6	-0.14

Ebersole [37]	Albumin	OTHERS	30/50	NS (mg/ml)	0.800	72.5	68.0/ 80.0	85.0/ 60.0	3.4/ 0.4	8.5	0.48
Bejeh-Mir [50]	nitrate		28/14	123.6 (NSp)	1.000	95.2	92.9/ 96.4	92.9/ 96.4	26.0/ 0.1	351.0	0.89
Bejeh-Mir [50]	nitric_oxide		28/14	246.6 (NSp)	1.000	95.2	92.9/ 96.4	92.9/ 96.4	26.0/ 0.1	351.0	0.89
Samani [53]	nitric_oxide		27/17	92.0 (mg)	0.875	86.4	76.5/ 92.6	86.7/ 86.2	10.3/ 0.3	40.6	0.69
Bejeh-Mir [50]	nitrite		28/14	12348.0 (NSp)	1.000	95.2	92.9/ 96.4	92.9/ 96.4	26.0/ 0.1	351.0	0.89

2.5. DISCUSSION

2.5.1. Quality of Diagnostic Accuracy Studies in Saliva and Heterogeneity Observed

The use of a dual process (computerised and manual) for the selection of articles enabled us to observe that the diagnostic accuracy literature on salivary molecular biomarkers in periodontitis represents around 2.5% of the literature obtained in our search. Subsequently, we found that nearly 40% of the diagnostic accuracy studies in saliva did not meet the methodological demands previously described for inclusion in a systematic review of diagnostic accuracy research [54].

The first interesting finding we observed about the previous systematic review on GCF biomarkers (Objective 1) is that their ability to diagnose periodontitis is understudied equally in both GCF and saliva. This fact is surprising because, logically, the diagnostic capacity of biomarkers should initially be more investigated in the gingival sulcus, which is the area where the disease develops; to subsequently investigate the extrapolation of the findings from GCF to saliva.

Regarding the methodological quality of the papers assessed using the QUADAS-2 tool [25], about 84% of the included articles were theoretically catalogued as case-controls, which are at higher risk than cohort studies [55]. However, from a quality point of view, there is a very positive aspect in practically all of the papers included, the presence of a single-gate design. This design has two main characteristics: 1) a single set of inclusion criteria were used for all subjects; and 2) most of the patients, both control and target groups, were examined clinically in the same setting, applying the same clinical parameters defined in the reference standard (total verification) [56]. The practice of the total verification of diagnosis using a reference standard is a very positive methodological feature, as it contributes to controlling possible imbalances between sensitivity and specificity values [57].

The authors did not apply pre-specified thresholds of the salivary molecular biomarkers in about 89% of the articles but instead selected

the threshold to optimise sensitivity and specificity. It is well-known that threshold optimisation is associated with an overestimation of the performance of the test [25], which can be controlled by a validation analysis (internal or external) [58]. However, most of the papers in the present review (15 of 18) did not perform any validation analysis.

The reference standard correctly classified the periodontal condition without knowledge of the results of the salivary biomarker in 89% of the included studies, although calibration aspects were cited in only 39% of the series. The time between the standard reference and the index test was adequately provided in 61% of the papers. An “objective interpretation” of the biomarker results was assumed in all cases since they were objectively quantifiable molecules. Due to the broad nature of the review question raised in this study, concerns about applicability were rated as "low" in practically all the included articles (except for two that applied the CPI diagnostic system).

Although curiously, it is not an evaluable aspect in the QUADAS-2 tool [25], we believe that one of the main methodological problems detected in the included studies is the small sample sizes used. Approximately 47% and 37% of the series had ≤30 control and target subjects, respectively. It is well-known that these sample sizes will produce unreliable results of diagnostic accuracy because the variation in the classification of a single subject provokes a substantial modification of in the sensitivity or specificity parameters (>3.3%).

About the heterogeneity observed in the papers included in this systematic review, as in Objective 1, the variability of the definitions of the clinical phenotype of periodontitis in diagnostic accuracy studies had to be accepted to carry out this systematic review. Similar to Objective 1, the reference standard for the diagnosis of the periodontal condition was considered to be the “gold standard”, which means it is 100% sensitive and specific [56]. It is well known that the reference standard is not "perfect" [6], and thus the possibility of verification errors that will condition the performance parameters exists, with a tendency to underestimate (because possible errors are unrelated) [56].

In order to ensure an adequate spectrum of patients and to control the risk of performance overestimations [56], this study considered different control (e.g., subjects with good periodontal health or those with good periodontal health and gingivitis) and target patients (e.g., different degrees of the extent and severity of chronic periodontitis).

All the articles included in the present review performed a patient-based assessment of the periodontitis status because the whole saliva represents a pooled sample with contributions from all periodontal sites [59,60]. In contrast to assessments of GCF, the disadvantage of salivary analyses is their inability to detect sites of disease activity [61,62]. We also observed that in a high percentage of series, around 63%, the samples analysed were of unstimulated saliva. The use of unstimulated saliva is preferred over other methods (stimulated saliva or rinsing), as it avoids a possible dilution of the biomarkers of interest, which could affect their quantification [6].

2.5.2. Accuracy of Biomarkers in Saliva for the Diagnosis of Periodontitis

After reviewing the literature, we found only one systematic review on the accuracy of salivary biomarkers for the diagnosis of periodontitis, namely the study published by de Lima et al. [18]. As a conclusion to their paper, these authors noted that there is currently limited evidence to confirm the diagnostic ability of salivary biomarkers in the clinical assessment of periodontal disease. In this sense, the present systematic review significantly improves the evidence on the topic reported by de Lima et al. [18] by analysing a higher number of studies. Firstly, the use of a higher number of search words, including specific names of biomarkers, and the application of an automated process for the search and selection of articles could increase the possibility of capturing a more significant number of candidate papers. Secondly, we observed differences between the two systematic reviews in the inclusion criteria applied concerning the reference standard, which could affect the number of selected papers. De Lima et al. [18] excluded those studies in which the periodontal status was evaluated by only one of the available clinical measurements

(either PPD or CAL) or the Community Periodontal Index. In contrast, we decided that these studies were initially included in the present review in order to later evaluate them methodologically. Thirdly, our review included a considerable number of new articles published after February 2015.

We want to justify why we applied less strict inclusion criteria about the reference standard. Initially, we agreed with de Lima et al. [18] that the use of one or another clinical parameter could affect prevalence in the diagnosis of periodontitis. However, even using both clinical parameters, the diagnostic criteria for periodontitis applied in each paper are heterogeneous; this fact by itself conditioned the prevalence of periodontitis. Furthermore, in our case, the possible error of the (imperfect) reference standard for the diagnosis of periodontitis is unrelated to the errors of the biomarker test (clinical phenotype *versus* biological phenotype). This premise means that it is very difficult to predict if there is likely to be biased about biomarker accuracy and its likely direction [56]. Even if this heterogeneity between studies related to the reference standard is assumed, from a diagnostic accuracy point of view, it is very important to point out the absence of intra-study heterogeneity. Unlike other diseases, in the clinical diagnosis of periodontitis, all the subjects of both groups (control and target condition) are verified by the same reference standard in all studies. Consequently, we decided to apply less rigid inclusion criteria about the reference standard in order to include a higher number of papers and data for later analysis.

After studying the paper of de Lima et al. [18] in-depth, we believe that, although the authors claim that it is a meta-analysis, a meta-analytical examination of a specific salivary biomarker was not carried out [32,63]. Accordingly, de Lima et al. [18], conditioned by the available data, were limited to making a graphic representation in the ROC space of all the biomarkers included without summary estimates, which is not a significant contribution concerning coupled forest plots [32,63]. Given this essential methodological consideration, the present systematic review is, to the best of our knowledge, the first meta-

analysis on the accuracy of specific salivary biomarkers for the diagnosis of periodontitis.

The first important finding derived from the present study is that there are a large number (up to 32) of individual salivary biomarkers of a different nature that have been studied from an accuracy perspective. These biomarkers are of different types, covering the three main biological phases of periodontitis (inflammatory, connective tissue degradation, and bone-turnover) [6]. However, 84% of biomarkers were evaluated in one or two articles, whereas only 16% (five biomarkers) were analysed in at least three publications. Our impression is that there is scientific interest in the initial discovery of salivary biomarkers for the diagnosis of periodontitis. There is, however, a lack of interest or difficulty in obtaining funding for validating the initial results, which is a necessary step if the diagnostic accuracy of a salivary biomarker is to be considered reliable.

Based on our findings, enzymes are the most frequently researched individual salivary biomarkers, representing 44.2% of the classifications evaluated, followed by inflammatory mediators and tissue-breakdown products (27.9% and 22.1%, respectively). Considering the number of articles included, the most studied were MMP8 and IL1beta, followed by IL6 and MMP9, and Hb, which allowed their quantitative analysis. Based on the meta-analytic analyses and considering the sensitivity and specificity interpretations used by some authors [64], all these biomarkers showed a good capability to detect periodontitis condition (>70%); MMP9 and IL1beta also showed a good capability to detect the non-periodontitis condition (around 80%).

It is well-known the methodological difficulties associated with the detection and quantification of cytokines in saliva due to the low concentrations of these biomarkers [65]. However, in the present meta-analysis, IL1beta was associated with the best estimated sensitivity and specificity values for the diagnosis of periodontitis (close to 80%), followed by IL6 (72% sensitivity and 73% specificity). These results confirm the qualitative findings previously described by de Lima et al.

[18] and demonstrate parallelism with other systematic reviews not focused on the diagnostic accuracy field, such as the one published by Gomes et al. [14].

MMP8 is regarded as one of the promising candidates for diagnosing and predicting the progression of periodontitis [66]. In the present meta-analysis, and terms of estimated sensitivity, MMP8 was the second best salivary biomarker, with a value of 72.5%; these results are in line with those reported in the systematic review by de Lima et al. [18]. However, these meta-analytical results differ from the accuracy data associated with the aMMP-8 PoC/chair-side technologies found by Alassiri et al. [10], who obtained that these tests have diagnostic sensitivity and specificity values of 76–90% and 96%, respectively, corresponding to an odds ratio of >72. In order to justify the discrepancy, and after checking the references on salivary MMP8 that supported the statements of Alassiri et al. [10] we were able to verify that these original research studies [67,68] were not included in the present systematic review, because they did not meet any of the previously established inclusion criteria.

Curiously, MMP9 was the biomarker with the worst estimated sensitivity and the best estimated specificity (70.3% and 81.5%, respectively). Interestingly, the present study also revealed that another biomarker, Hb, had a similar sensitivity value to MMP8 and IL6 (72%), and even a better specificity value (75%, *versus* 70.5% and 73%, respectively).

The diagnostic accuracy results of MMP8 in saliva showed biological coherence if we consider the meta-analysis of this biomarker in GCF previously performed by our group [69]. Reasonably, a biomarker that has a good capability in GCF for the detection of periodontitis should maintain this level of accuracy in saliva, although it probably decreases; indeed, this was what we observed for MMP8 (sensitivity decreased from 77% to 72.5%, and specificity from 92% to 70.5%). Derived from these findings and comparatively with the GCF, we can deduce that salivary MMP8 will have more difficulties in its ability to detect the control condition (non-periodontitis) than the target

condition (periodontitis); this could probably be a consequence of the influence of other factors that can condition the salivary sample in control conditions. Unfortunately, this interesting comparison cannot be made for the rest of the biomarkers meta-analysed in saliva, because they did not coincide with those meta-analysed in GCF.

Concerning other less researched biomarkers, the results obtained should be interpreted with caution. However, we consider it essential to highlight the excellent accuracy data of five salivary biomarkers: cysteine had an ACC value of 97% [48]; MIP1alpha, an ACC of 94% [49]; and nitrate, nitric oxide and nitrite, ACC values of 95% [50].

Although the negative impact that smoking may have on the accuracy of salivary biomarkers in the diagnosis of periodontitis has been highlighted, for example for MMP8 [70], almost none of the studies included in the present review evaluated it as a possible covariable on the accuracy data. Only Nomura et al. [45] adjusted their predictive models for LDH and Hb to covariates such as age, the number of remaining teeth and smoking habits, although no improvement was observed in the sensitivity and specificity data with the latter covariable.

2.5.3. Implications for Practice

Recently, Tonetti et al. [3] recognised that biomarkers could play a crucial role in the early detection of periodontitis and assessments of its severity grade. In this sense, a salivary diagnostic tool can serve as a non-invasive, sensitive, specific and useful test as adjuncts for patient care and maintenance, i.e., the early identification of increased tissue inflammation [6]. It can also help in screening large populations for the detection of periodontitis [60].

If the natural progression of periodontitis substantially complicates the discovery of biomarkers in GCF [9], it is reasonable to think that this difficulty will be even higher in saliva. Although the advantages associated with the use of salivary samples are evident [6], there are also shortcomings in their use that must be considered. The analytes in saliva are present in lower amounts, meaning that assays need to be

highly sensitive [17]. Saliva contains several proteases, which potentially degrade protein biomarkers [71], and the presence of mucins and cell debris makes saliva a challenging fluid to work with [17]. Additional confounders include low flow rates due to dehydration, drug administration, systemic diseases or physiological conditions that can affect/limit saliva collection, as well as diurnal variations [6,72]. However, it is essential to note that it is likely that within-subject variations in the concentration of salivary biomarkers can be overcome for diagnostic purposes if a biomarker with substantial inter-group differences is chosen [8].

A first-line test also called a triage test, may be clinically useful even when the sensitivity or specificity is not high, depending on the steps that will be taken after testing [57]. Focusing on the two biomarkers that were more researched (and therefore more reliable), and considering the sensitivity and specificity interpretations used by some authors [64], we found in our review that both MMP8 and IL1beta showed good diagnostic capability to detect periodontitis [64]; although IL1beta showed higher estimated values of sensitivity and specificity. In our opinion, these results are quite positive if we take into account the broad spectrum considered in both the control condition (periodontal health and gingivitis) and the target condition (different extents and severity of periodontitis).

In terms of usefulness or effectiveness of the biomarker, considering a 45% prevalence of periodontitis [46,47] and if IL1beta and MMP8 tests in saliva were used as a first line test, there would be 67% and 63%, respectively, of the total of positive tests in which the test could correctly detect disease in the initial screening; on the other hand, the tests could correctly detect the condition of non-periodontitis in 79% and 75%, respectively, of the total of negative tests.

2.5.4. Implications for Future Research

Surprisingly, the present systematic review reveals a lack of biological concordance in the accuracy field of salivary biomarkers for the diagnosis of periodontitis. This finding is supported by the fact that

the biomarkers that are most investigated in saliva are not those most investigated in GCF (Objective 1), except MMP8.

Secondly, despite the great interest shown by the scientific community on the topic, more high-quality evidence is needed on the diagnostic accuracy of salivary biomarkers in periodontitis, with the focus mainly on the analysis of large series [58].

The main limitation of this review was that it did not have a higher number of series with large sample sizes in order to obtain more robust results from meta-analyses.

According to the latest methodological protocols [58], the diagnostic accuracy studies have to include a validation analysis and should also consider the influence of variables such as the presence of gingivitis in the control condition, smoking and the presence of systemic diseases in order to refine the diagnostic accuracy results. The organisation of an International Consortium for Salivary Biomarkers of Periodontitis (ICSBP) has even been proposed, which would ensure the implementation of standardised protocols for clinical research [8]. The existence of more considerable evidence on the diagnostic accuracy of salivary biomarkers would allow more demanding systematic reviews and meta-analyses of single or combined biomarkers. These should involve threshold and heterogeneity analyses, which are advanced evaluations that could not be carried out in the present study due to the limited literature available.

Based on the meta-analytic analysis, MMP8, MMP9, IL1beta, IL6 and Hb were salivary biomarkers with good capability to detect periodontitis in systemically healthy subjects; MMP9 and IL1beta also showed good capability to detect the non-periodontitis condition. MMP8 and IL1beta were the most researched salivary biomarkers in the diagnostic accuracy field, both showing clinically fair effectiveness for the diagnosis of a broad spectrum of periodontitis.

Other molecules, such as cysteine, MIP1alpha and nitric oxide (and its related-metabolites), were identified as promising salivary biomarkers, but more research is needed to confirm these findings.

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OBJECTIVE 3

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Objective 3. Cytokine-based Predictive Models to Estimate the Probability of Chronic Periodontitis: Development of Diagnostic Nomograms

3.1. ABSTRACT

Aims: Although a distinct cytokine profile has been described in the gingival crevicular fluid (GCF) of patients with chronic periodontitis, there is no evidence of GCF cytokine-based predictive models being used to diagnose the disease. Our objectives were: to obtain GCF cytokine-based predictive models; and develop nomograms derived from them.

Material and Methods: A sample of 150 systemically healthy participants was recruited, including 75 periodontally healthy controls and 75 subjects affected by chronic periodontitis. Sixteen mediators were measured in GCF using the Luminex 100™ instrument: GMCSF, IFNgamma, IL1alpha, IL1beta, IL2, IL3, IL4, IL5, IL6, IL10, IL12p40, IL12p70, IL13, IL17A, IL17F and TNFalpha. Cytokine-based models were obtained using multivariate binary logistic regression. Models were selected for their ability to predict chronic periodontitis, considering the different role of the cytokines involved in the inflammatory process and then, adjusted by smoking status.

Results and Conclusions: The outstanding predictive accuracy of the resulting smoking-adjusted models showed that IL1alpha, IL1beta and IL17A in GCF are outstanding biomarkers for distinguishing systemically healthy patients with chronic periodontitis from periodontally healthy individuals (AUC=0.973, 0.963 and 0.937,

respectively). IL1alpha and IL1beta were associated with percentages of sensitivity and specificity above 90%, while IL17A showed 89% values for both parameters. The predictive ability of these pro-inflammatory cytokines was increased by incorporating IFNgamma and IL10, specifically in the case of IL17A (from AUC=0.937 to 0.974). The nomograms revealed the amount of periodontitis-associated imbalances between these cytokines with pro-inflammatory and anti-inflammatory effects in terms of a particular probability of having chronic periodontitis. The clinical implications of these biomarkers could include improved patient monitoring and the control of disease activity, although external validation studies are needed.

3.1.1. Keywords

Chronic periodontitis, gingival crevicular fluid, cytokines, inflammation, multiplex immunoassay, multivariate predictive modelling techniques, area under curve, accuracy, sensitivity, specificity, nomograms.

3.1.2. Declaration of conflict of interest

The doctoral candidate and the rest of the authors of this paper declare that they have no conflict of interest concerning the objectives proposed in this chapter.

3.1.3. Funding

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3.2. Introduction

Periodontal diseases are among the most common conditions affecting humans [1]. In 2010, severe periodontitis was estimated to be the sixth most prevalent disease globally, affecting 743 million people worldwide and with an age-standardised incidence of 701 cases per 100,000 person-years [2]. A recent prevalence study of adult periodontitis in the USA, with data from the 2009 and 2010 National Health and Nutrition Examination Survey, revealed that over 47% of individuals had periodontitis, which equates to 64.7 million adults [3,4]. During the recent 11th European workshop on periodontology, experts confirmed that the prevalence of periodontitis in Europe remains high, affecting more than 50% of the adult population and, in its severe forms, 11% of adults [5].

Periodontitis is not a "silent" problem: periodontal patients have a more reduced perception of their oral health and a worse quality of life compared to healthy individuals, with periodontal treatment improving the oral health-related quality of life of these people [6,7]. On the other hand, periodontitis is currently being connected bidirectionally to the pathogenesis of various systemic diseases and conditions such as diabetes [8]; coronary heart disease [9]; rheumatoid arthritis [10]; respiratory diseases [11]; pre-term low birth weights [12]; and dementia [13,14].

Traditional clinical measures are informative for evaluating the severity of periodontitis and the response to therapy, and these include: the presence of plaque or the level of oral hygiene; the gingival inflammation and bleeding upon probing; the pocket depth and suppuration; the clinical attachment level; and the radiographic bone loss [15]. Nevertheless, these clinical criteria are unable to determine current disease activity or the future risk of structure loss [16,17]. As a result, one of the significant challenges in the field of Periodontology is to determine biomarkers for screening and predicting the early onset of periodontitis or evaluating the disease activity as well as the efficacy of therapy (diagnostic or prognostic tests) [16,18].

The primary hallmark of periodontitis, namely the destruction of periodontal tissue, is widely accepted to be a result of a chronic inflammatory host immune response caused by a polymicrobial dysbiosis [19,20]. This host immune response is characterised by: 1) the infiltration of the gingival tissues by neutrophils, monocytes/macrophages and lymphocytes; and 2) the generation of high concentrations of mediators, including cytokines, chemokines, arachidonic acid metabolites and proteolytic enzymes [19,21]. The nature and extent of this host immune response are fundamental determinants of the susceptibility and progression of periodontitis [22].

Gingival crevicular fluid (GCF) is an exudate of the serum originating from the gingival plexus of blood vessels in the gingival connective tissue, close to the epithelium lining of the dentogingival space. With an increase in the severity of periodontal inflammation, the amount of GCF increases significantly. Additionally, its consistency transforms into an inflammatory exudate as it traverses the inflamed tissues, collecting bacterial and host molecules [23,24]. The collection of GCF is relatively non-invasive, and this sampling method has been shown to capture inflammatory and connective tissue breakdown mediators accurately [24].

With the establishment of enzyme-linked immunosorbent assay (ELISA) techniques, interleukin (IL) 1beta was the first cytokine to be measured explicitly in the gingival tissue of patients with chronic periodontitis [25]. Numerous papers since then have reported the measurement of cytokines in GCF, confirming that there is a distinct cytokine profile for patients with chronic periodontitis [18,26]. The results concerning which cytokines are most involved in chronic periodontitis are, however, generally contradictory, due to the lack of uniformity in the methodological design of the studies [26]. On the other hand, very few authors have examined the simultaneous presence of more than ten cytokines in GCF, and there is a failure to analyse a broader spectrum of biomarkers that may directly influence the local inflammatory response in periodontitis [27–29]. Moreover, there is no evidence in the literature of the development, validation, or updating of GCF cytokine-based predictive models for diagnosing periodontitis or

its prognosis using appropriate multivariate predictive modelling techniques [30]. As a consequence, it is quite clear that highly specific and sensitive biomarkers for monitoring periodontitis are still needed for the early and better detection of periodontal tissue destruction [18].

Accordingly, the objectives of the present cross-sectional study were to:

1) Compare the levels of 16 cytokines detected in GCF between systemically healthy individuals with periodontal health and patients with chronic periodontitis.

2) Obtain GCF cytokine-based predictive models that could be used to distinguish periodontal patients from periodontally healthy individuals.

3) Develop nomograms derived from GCF cytokine-based predictive models.

3.3. Material and Methods

3.3.1. Selection of Study Groups

A convenience sample of 150 eligible participants, comprising 75 periodontally healthy controls (control group) and 75 subjects affected by moderate to severe generalised chronic periodontitis (perio group), were recruited among 250 consecutive patients of the general population who were referred to the School of Medicine and Dentistry (Universidade de Santiago de Compostela, Spain) for an assessment of their oral health status between 2013-2015. Patients were selected if they fulfilled the following inclusion criteria: 1) age 30 to 75; 2) no medical history of diabetes mellitus or hepatic or renal disease, or other severe medical conditions or transmittable diseases; 3) no history of alcohol or drug abuse; 4) no pregnancy or breastfeeding; 5) no intake of systemic antimicrobials during the previous six months; 6) no intake of anti-inflammatory medication in the previous four months; 7) no routine use of oral antiseptics; 8) no presence of implants or orthodontic appliances; 9) no previous periodontal treatment; 10) smokers for a

minimum period of eight years; 11) smokers, who had stopped smoking less than five years before the sampling; 12) the presence of at least 18 natural teeth (Figure 1).

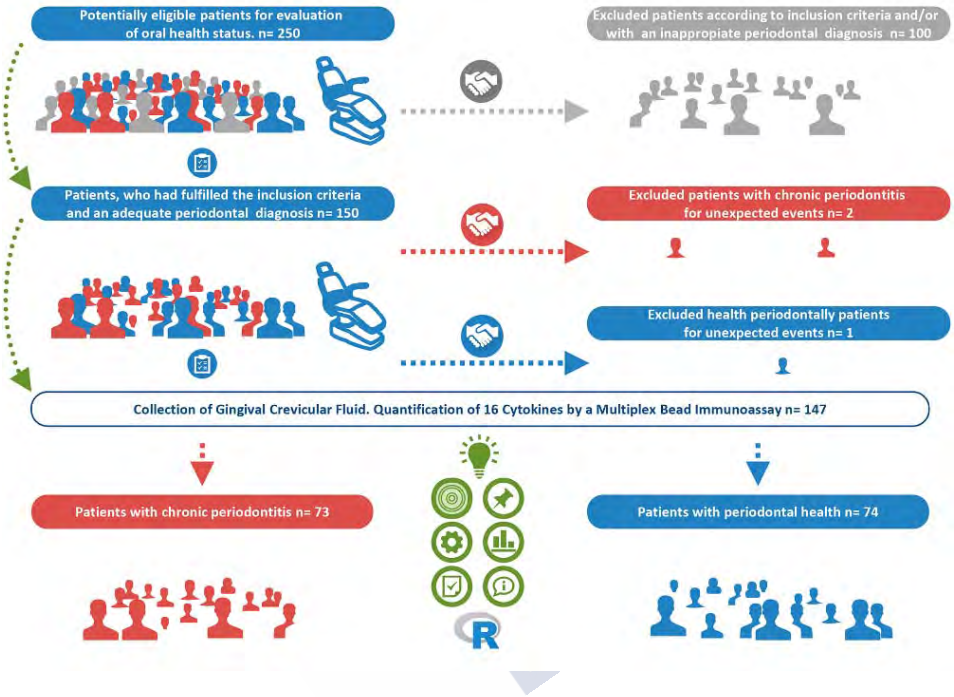

Figure 1. Selection of study groups. From the 250 potentially eligible patients, 100 subjects were excluded because they did not fulfil the inclusion criteria. One hundred fifty systemically healthy patients were included in the study, but for unexpected reasons, three subjects were excluded (2 periodontitis patients and one periodontally healthy).

One experienced dentist, who previously underwent a calibration exercise of the clinical parameters in a small group of patients, performed all the periodontal diagnoses. The probing pocket depth (PPD) and the clinical attachment level (CAL) were recorded throughout the mouth on six sites per tooth using a PCP-UNC 15 probe. The bleeding on probing (BOP) and the bacterial plaque level (BPL) were recorded for the full mouth on a binary scale (presence/absence) on six sites per tooth. Standardised radiographs of all teeth were obtained to assess the alveolar bone status.

The presence or absence of chronic periodontitis was based on clinical/radiographic information. The control group included periodontally healthy individuals with BOP <25%, no sites with a PPD ≥4 mm and no radiographic evidence of alveolar bone loss. The perio group patients were diagnosed as suffering from moderate to severe generalised chronic periodontitis based on the previously established criteria [31,32]. Smoking histories were obtained using a questionnaire, with information collected on smoking status (never, past or current), the years (months) of smoking and the number of cigarettes/day. All answers were reviewed with the subject by a member of the study team. Patients were defined as smokers if he/she was currently smoking and had been a smoker for at least eight years and as non-smokers if he/she had never smoked or quitted smoking longer than five years before the sampling.

This study was conducted according to the principles outlined in the Declaration of Helsinki (as revised in 2000) on experimentation involving humans [33]. Patients who agreed to participate in the research provided written informed consent. The study's protocol was approved by the Clinical Research Ethics Committee of Galicia (number 2015/006; Appendix S1). The transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (TRIPOD) guidelines and STARD guidelines for reporting diagnostic accuracy studies were applied [30,34]. The TRIPOD checklist is shown in Appendix S2.

3.3.2. Gingival Crevicular Fluid Sampling

The GCF collection took place one week after the initial examination, and the samples were obtained at the same time of day (in the afternoon, approximately 5-7 hours after toothbrushing). Before the sampling, the individual tooth site was isolated with cotton rolls, the supragingival plaque was carefully removed, and the site was gently air-dried with an air syringe. A paper strip (Periopaper, Amityville, NY, USA) was inserted into the gingival sulcus or periodontal pocket for 30 seconds. In cases of visible contamination with blood, the strips were discarded and new sites sampled. To ensure sample collection, the GCF volume was determined based on measurements of weighing the tubes

and strips before and after sampling using a high-precision weighing scale. All samples collected presented volumes of GCF ≥10 µl.

GCF samples from the periodontally healthy patients were collected and pooled from 20 sites from teeth in quadrants 1 and 3 (incisor, canine, first premolar, second premolar and molar). In the periodontal patients, subgingival samples were collected and pooled from the deepest PPD sites in each quadrant (a total of 20 non-adjacent proximal sites). Strips from each subject were placed into labelled tubes containing: 300 ml of 0.01M PBS with a pH of 7.2; and a protease inhibitor (Complete Mini, protease inhibitor cocktail tablets, Roche Applied Science, Indianapolis, IN, USA). To prevent evaporative losses, the GCF volume was determined based on immediate measurements of weighing the tubes and strips before and after the sample collection using a very sensitive scale [23].

After shaking for 20 minutes, the strips were removed and the eluates centrifuged for 5 minutes at 5800 g to remove plaque and cellular elements. The GCF samples were then frozen at -80ºC until further biochemical analysis.

3.3.3. Quantification of Cytokines in Gingival Crevicular Fluid Using Multiplexed Bead Immunoassays

A single investigator blinded to the clinical data performed the quantitative cytokine analyses. Cytokine levels in the GCF were determined using the human cytokine 16-plex Procarta immunoassay (Affymetrix, Inc., Santa Clara, CA, USA). Sixteen mediators were measured: 1) eight pro-inflammatory cytokines (granulocyte-macrophage colony-stimulating factor -GMCSF-, IL1alpha, IL1beta, IL6, IL12p40, IL17A, IL17F and TNFalpha); and 2) eight anti-inflammatory cytokines (IFNgamma, IL2, IL3, IL4, IL5, IL10, IL12p70 and IL13).

The assays were performed in 96-well filter plates following the manufacturer's instructions. Briefly, the filter plates were pre-wet with washing buffer, and the solution was aspirated from the wells using a handheld magnetic separator block (Millipore Corporation, Billerica, MA). Microsphere beads coated with monoclonal antibodies to the 16 different target analytes were added to the wells. Standards and samples were pipetted into the wells and incubated overnight at 4°C. The wells were then washed, again using a handheld magnetic separator block (Millipore Corporation), and a mixture of biotinylated secondary antibodies was added. After incubation for 30 minutes, streptavidin conjugated to the fluorescent protein phycoerythrin (PE)-conjugated streptavidin was added to the beads and incubated for 30 minutes. After washing to remove the unbound reagents, a reading buffer (Affymetrix, Inc) was added to the wells, and the beads (minimum of 100 per analyte) were analysed using the Luminex 100™ instrument (Luminex Corporation, Austin, Texas, USA). All the samples were run in duplicate.

The Luminex 100™ monitored the spectral properties of the beads to distinguish the different analytes while simultaneously measuring the amount of fluorescence associated with PE-streptavidin, which is reported as the median fluorescence intensity (MFI). The concentrations of the unknown samples (antigens in the GCF samples) were: 1) estimated from the standard curve using a 5PL algorithm and the Luminex IS 2.3 and xPONENT 3.1 softwares (Luminex Software, Inc.); 2) expressed as pg/ml adjusting for the dilution factor. The concentration ranges for each biomarker analysed were: GMCSF, 0.53-55,050 pg/ml; IFNgamma, 0.02-6,650 pg/ml; IL1alpha, 0.34-28,800 pg/ml; IL1beta, 0.09-23,150 pg/ml; IL2, 0.04-13,700 pg/ml; IL3, 0.19-26,500 pg/ml; IL4, 0.10-29,250 pg/ml; IL5, 0.04-17,800 pg/ml; IL6, 0.10-27,200 pg/ml; IL10, 0.04-10,050 pg/ml; IL12p40, 0.14-27,350 pg/ml; IL12p70, 0.26-18,050 pg/ml; IL13, 0.34-23,700 pg/ml; IL17A, 0.36-30,900 pg/ml; IL17F, 0.25-34,700 pg/ml; and TNFalpha, 0.21-16,800 pg/ml.

Samples below the detection limit (DL) of the assay were recorded as DL/2 [35], while samples above the upper limit of quantification of the standard curves were assigned the highest value of the curve.

3.3.4. Statistical Analysis

The statistical analyses were performed using the R software, version 3.4.3 [36].

3.3.4.1. Comparison of Clinical Characteristics between Periodontally Healthy Individuals and Patients with Chronic Periodontitis

Univariate tests to detect differences in the clinical characteristics between the control and perio groups were performed. The normality assumption of the quantitative variables was checked by the Shapiro-Wilk test, with the result being that the normality hypothesis was not valid in all the cases. Consequently, the Mann-Whitney U test was used to compare the quantitative clinical characteristics between both groups (age, number of teeth and the clinical parameters BPL, BOP and CAL in the full mouth and the sampled sites).

The Fisher's exact test was used to assess the association between the qualitative variables (gender and smoking habit) between both study groups. The significance level applied was $p < 0.05$.

3.3.4.2. Comparison of GCF Cytokine Levels between Periodontally Healthy Individuals and Patients with Chronic Periodontitis

After applying the Shapiro-Wilk test, and because the distributions of the cytokines were skewed, we used logarithmically transformed values ($\log_2$) for the statistical analyses. Quantitative data on the GCF cytokine levels were expressed as medians and interquartile ranges (IQR). The Mann-Whitney U test was used to compare the GCF cytokine levels between control and perio groups.

3.3.4.3. Multivariate Predictive Modelling of Chronic Periodontitis Based on GCF Cytokine Levels: Model Selection and Validation, and the Development of Nomograms

In the calculation of the sample size, estimating a predetermined AUC value of 0.850 with a given marginal error of 0.07 and 95% confidence level, the required sample size was around 75 subjects in each clinical condition [37].

Spearman correlations between cytokines were calculated and used as an orientation for model building, in order to prevent redundancies and possible collinearity between cytokines with similar biological effects. Cytokine-based models were selected for their biological significance, their capacity to predict chronic periodontitis and their statistical validity. The biological criteria to select the predictor cytokines are based on their importance level in the inflammatory process, and particularly the different role of pro-inflammatory and anti-inflammatory cytokines.

Models were obtained using binary logistic regression and initially selecting one pro-inflammatory cytokine as a predictor variable. In order to test whether the predictive ability of pro-inflammatory cytokines can be increased by incorporating cytokines with anti-inflammatory effects, two-variable models combining these different mediators were analysed. Resulting models were adjusted individually in relation to the "smoking status" (a non-smoking status was established as the reference).

The statistical criterion applied for model selection was the capacity of each GCF cytokine-based model to discriminate the presence of chronic periodontitis, that was assessed with the Epi package (version 2.12) and using the receiver operating characteristic (ROC) curve [38]. This curve was created by plotting the true positive rate (TPR; sensitivity) *versus* the false positive rate (FPR; 1-specificity) at various threshold settings of the analyses. The area under the curve (AUC), which is the C index, was regarded as a measure for the discriminative capacity of the model and provides a scale from 0.5 to

1.0 (with 0.5 representing random chance and 1.0 indicating perfect discrimination) with which to compare the ability of a biomarker to detect a positive result [39]. Note that models with an AUC value equal to or higher than 0.70 are typically considered to be acceptable predictive models [40]. The calculation of the AUC values and their corresponding 95% confidence intervals (CIs) by bootstrapping were performed using the pROC package, version 1.10.0 [41]. Those models that presented higher AUC values were selected.

Of the selected cytokine-based models, using the pROC package and bootstrapping, numerous classification measures such as the accuracy (ACC), the sensitivity (SENS), the specificity (SPEC), the positive predictive value (PPV), and the negative predictive value (NPV) were obtained by setting an optimal threshold, as well as their corresponding 95% Cis [41]. The best cut-off value for each model was determined so that the percentage of correct predictions was the maximum. As a single indicator of diagnostic performance, the diagnostic odds ratio (DOR) was calculated as the ratio of the odds of positivity in the diseased patients relative to the odds of positivity in those with no disease [42].

The Hosmer-Lemeshow test was applied to the selected cytokine-based models, using the Resource Selection package, version 0.3-0 [43]. This test is a calibration measure, which is significant for poorly calibrated models [44]. Calibration curves of these models were constructed graphically using the rms package, version 5.0-1 [45] to assess the agreement between the actual outcomes and the predicted probabilities of chronic periodontitis. In a well-calibrated model, the predictions should fall on a 45-degree diagonal line [44].

The nomograms were built based on the results of multivariable analyses using the rms package [45]. A nomogram maps the predicted probabilities into points on a scale from 0 to 100 in a user-friendly graphical interface. The total points accumulated by the various covariates correspond to the predicted probability of disease for a patient [46].

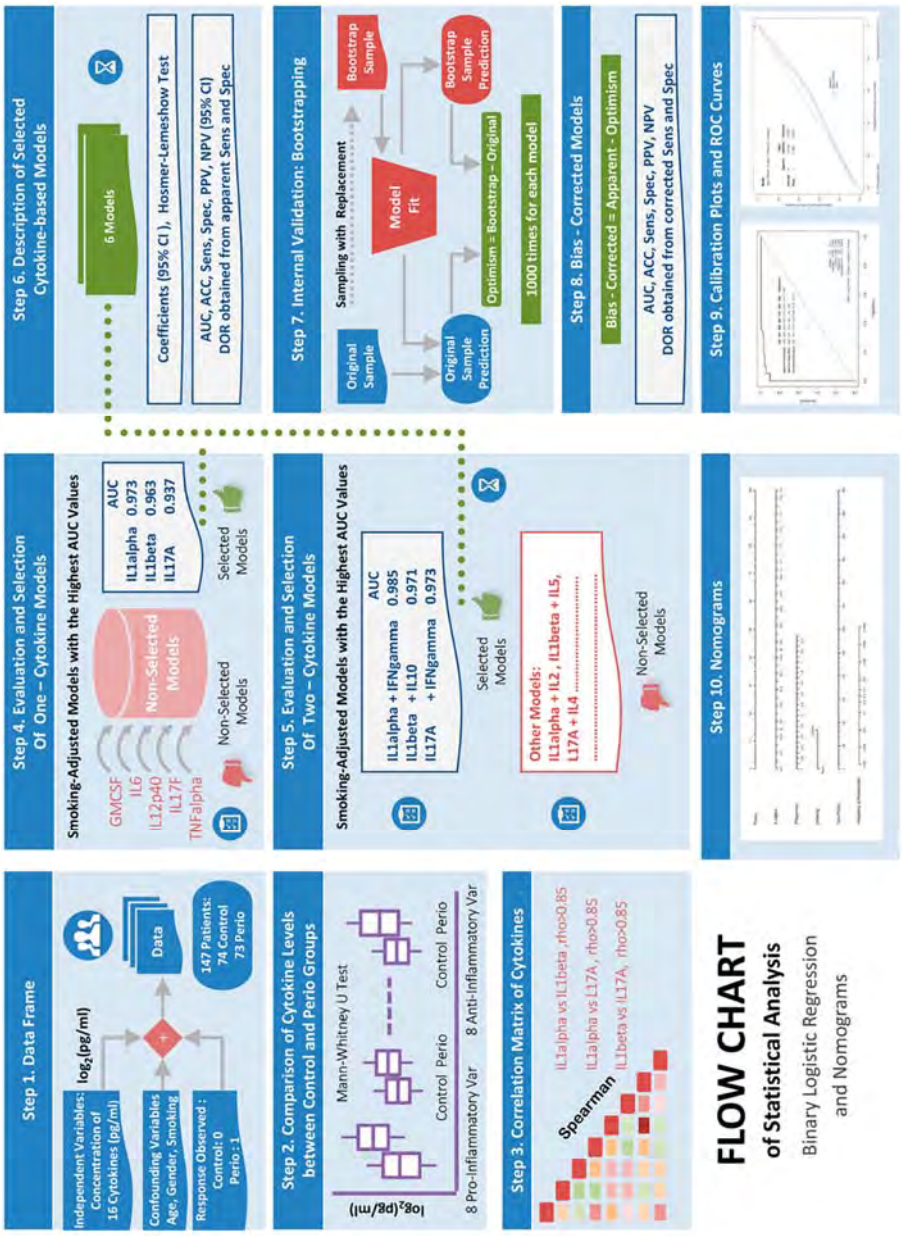

Figure 2. Flow chart of statistical analysis: binary logistic regression and diagnostic nomograms.

3.3.4.4. Internal Validation

Bootstrap methods were used to test for possible overfitting by determining optimism values on the discrimination, classification and calibration measures. The bootstrap analysis was replicated on 1000 different samples of the same sample size drawn with replacements from the original sample. Optimism, which is a measurement of internal model validation that refers to the absolute magnitude of bias, equals the difference between respective statistics of the bootstrap sample (bootstrap performance) and the bootstrap model in the original sample (test performance) [47,48].

Bias-corrected (bc) AUC, all the classification measures (bc-sensitivity, bc-specificity, bc-PPV, bc-NPV), bc-calibration measures were calculated as their corresponding apparent measures derived from the entire original sample minus optimism [47,48]. In terms of the bc-DOR, these ratios were calculated from the values of bc-sensitivity and bc-specificity. Figure 2 shows the flow chart of statistical analysis: binary logistic regression and diagnostic nomograms.

3.4. RESULTS

3.4.1. Comparison of Clinical Characteristics between Periodontally Healthy Individuals and Patients with Chronic Periodontitis

Of the 150 patients, who had fulfilled the inclusion criteria and adequate periodontal diagnosis, three patients were excluded for unexpected events (Figure 1). Of the 147 patients taking part in the study, who had a mean age of 48.37 ± 11.55 years, 62 were male and 85 female. The mean ages of patients of control and perio groups were 45.65 ± 12.37 and 51.12 ± 10.01 years, respectively (p=0.005; Table 1).

Table 1. Age, gender, smoking habit and clinical characteristics associated with the periodontal status in the control and perio groups.

CLINICAL PARAMETERS	STUDY GROUPS		
	Control group (n=74)	**Perio group (n=73)**	***P* Value**
Age (years)	45.65 (12.37)	51.12 (10.01)	0.005
Gender			
Male	31	31	NS
Female	43	42	
Smoking habit[1]			
Non-smokers	61	32	<0.001
Smokers	13	41	
Cigarettes/day (no.)	8.08 (4.44)	15.20 (7.94)	0.001
Months of smoking(no.)	236.38 (155.91)	320.78 (109.03)	NS
No. of teeth	26.72 (3.25)	25.55 (4.00)	NS
Full mouth			
BPL (%)	26.41 (18.66)	53.08 (26.77)	<0.001
BOP (%)	15.05 (6.61)	51.1 (20.07)	<0.001
PPD (mm)	2.11 (0.27)	3.49 (0.65)	<0.001
CAL (mm)	2.36 (0.46)	4.25 (1.12)	<0.001
Sampled sites			
BOP (%)	10.11 (10.24)	66.9 (23.93)	<0.001
PPD (mm)	2.23 (0.22)	5.65 (0.89)	<0.001
CAL (mm)	2.31 (0.27)	6.05 (1.12)	<0.001

Values are means (standard deviations) and the number of subjects.

Concerning smoking status, the number of smoker patients was significantly higher in the perio group than in the control group (41 and 13 patients, respectively; $p<0.001$). The analysis of clinical variables related to oral health status showed that, in comparison with the control group, the perio group had significantly higher values of BPL, BOP, PPD and CAL at both the full mouth and sampling site levels ($p<0.001$; Table 1).

3.4.2. Comparison of GCF Cytokine Levels between Periodontally Healthy Individuals and Patients with Chronic Periodontitis

Before the logarithmic transformation, the raw variables were very skewed, with few individuals having very large values, which makes it difficult to distinguish between individuals with small to moderate cytokine levels. After the logarithmic transformation, the individuals were more regularly spread in the possible values of the transformed variables.

The levels of all the pro-inflammatory cytokines (GMCSF, IL1alpha, IL1beta, IL6, IL12p40, IL17A, IL17F and TNFalpha) were significantly higher in the perio group compared to the control group (p<0.001, for all comparisons; Table 2A).

Table 2A. Concentrations (log_2 pg/ml) of eight pro-inflammatory cytokines In both study groups

Pro-inflammatory Cytokine	Concentration in GCF (log_2 pg/ml) Median (IQR)		*P* Value
	Control group	Perio group	
GMCSF	7.24 (1.21)	7.95 (1.48)	<0.001
IL1alpha	14.93 (0.88)	17.18 (1.66)	<0.001
IL1beta	11.50 (0.99)	14.13 (1.35)	<0.001
IL6	7.28 (1.95)	8.29 (2.00)	<0.001
IL12p40	3.12 (0.97)	4.17 (0.92)	<0.001
IL17A	3.00 (2.02)	4.86 (1.27)	<0.001
IL17F	2.04 (1.52)	3.37 (1.71)	<0.001
TNFalpha	2.86 (1.69)	4.55 (0.97)	<0.001

Table 2B. Concentrations (log_2 pg/ml) of eight anti-inflammatory cytokines in both study groups

Anti-inflammatory Cytokine	Concentration in GCF (log_2 pg/ml) Median (IQR)		*P* Value
	Control group	Perio group	
IFNgamma	2.41 (1.18)	3.36 (1.39)	0.001
IL2	3.42 (1.13)	3.97 (0.70)	<0.001
IL3	5.60 (1.07)	6.64 (1.88)	<0.001
IL4	2.74 (2.44)	3.71 (2.58)	<0.001
IL5	3.21 (1.30)	3.62 (0.83)	NS
IL10	2.64 (1.57)	3.10 (2.08)	NS
IL12p70	3.17 (3.05)	3.86 (1.97)	NS
IL13	4.90 (2.88)	5.06 (3.51)	NS

Regarding the anti-inflammatory cytokines, only four mediators (IFNgamma, IL2, IL3 and IL4) presented significantly higher concentrations in the Cigarettes group ($p<0.001$, for all comparisons). The increase in the concentrations of these anti-inflammatory cytokines was lower than that detected for the pro-inflammatory cytokines, except for IL3 (Table 2B). Boxplots for each cytokine in both study groups are given in Appendix S3.

3.4.3. Multivariate Predictive Modelling of Chronic Periodontitis Based on GCF Cytokine Levels: Model Selection and Validation, and the Development of Nomograms

For multivariate predictive analysis, we had a total of 147 participants and 73 outcome events. A description of the relation between cytokine levels is given in Appendix S4, using their Spearman correlations. Almost all correlations between cytokines, both pro-inflammatory and anti-inflammatory, were positive. The interpretation is that when periodontitis-associated inflammation was present, all cytokines presented larger values. Additionally, we observed more significant correlations between pro-inflammatory cytokines. Particularly, strong positive correlations were detected between some very relevant pro-inflammatory cytokines, IL1alpha, IL1beta and IL17A (rho>0.85). Note also that in the given boxplots these cytokines showed the biggest differences between both study groups.

Respect to the one-cytokine models adjusted by “smoking”, the pro-inflammatory cytokines IL1alpha, IL1beta and IL17 were the predictors that showed higher AUC values (0.973, 0.963, 0.937, respectively). Regarding the two-cytokine models adjusted by “smoking”, the incorporation of certain anti-inflammatory cytokines improved the AUC values of the best models based on a pro-inflammatory cytokine, especially that of IL17A (from 0.937 to 0.974). These two-cytokine models were: IL1alpha + IFNgamma, IL1beta + IL10 and IL17A + IFNgamma. The description of these six models, as well as their corresponding discrimination measures, are detailed in Table 3.

Table 3. Description of the six smoking-adjusted models based on cytokines.

Cytokine-based Models	AUC	bc-AUC
-50.32 + 3.13 **IL1alpha +** 1.78 **SmokingCurrent**	0.973	0.971
-27.72 + 2.13 **IL1beta** + 1.72 **SmokingCurrent**	0.963	0.962
-7.60 + 1.82 **IL17A** + 1.86 **SmokingCurrent**	0.937	0.934
-71.38 + 4.62 **IL1alpha** - 1.14 **IFNgamma** + 2.04 **SmokingCurrent**	0.986	0.983
-28.81 + 2.33 **IL1beta** - 0.50 **IL10** +1.70 **SmokingCurrent**	0.971	0.967
-12.37 + 5.02 **IL17A** - 3.16 **IFNgamma** + 2.98 **SmokingCurrent**	0.974	0.971

There are included apparent and bias-corrected AUC values.

The IL1beta model presented the highest bc-ACC percentage (93.0%; bc-sensitivity=92.1%; bc-specificity=93.9%; bc-DOR=183.0), followed by the IL1alpha (91.5%; bc-sensitivity=92.7%; bc-specificity=90.4%; bc-DOR=120.7), and IL17A (88.0%; bc-sensitivity=88.2%; bc-specificity=87.9%; bc-DOR=55.0).

In relation to two-cytokine models, the IL1alpha + IFNgamma model and IL1beta + IL10 model showed similar bc-ACC percentages (93.7% and 93.5%; bc-sensitivity=91.7% and 93.3%; bc-specificity=95.7% and 93.8%); while the IL17A + IFNgamma model had lower values of bc-ACC (91.2%; bc-sensitivity=89.1%; bc-specificity=93.3%). The bc-DOR values for these three models were 249.3, 211.7 and 115.6, respectively. Apparent and bias-corrected classification measures of the six cytokine-based models are described in Table 4. Additional information on these models and their corresponding performance measures are shown in Appendices S5 and S6.

Table 4. Apparent and bias-corrected measures of discrimination and classification of the six smoking-adjusted models based on cytokines.

Smoking-adjusted Model	ACC (%)	SENS (%)	SPEC (%)	PPV (%)	NPV (%)	DOR
IL1alpha	93.2 91.5	94.5 92.7	91.8 90.4	92.1 90.5	94.5 92.9	195.5 120.7
Il1beta	93.8 93.0	93.1 92.1	94.5 93.9	94.5 93.8	93.4 92.4	238.0 183.0
IL17A	89.1 88.0	89.0 88.2	89.1 87.9	89.0 87.8	89.2 88.5	67.0 55.0
IL1alpha + IFNgamma	95.2 93.7	93.1 91.7	97.2 95.7	97.1 95.6	93.5 92.3	489.5 249.3
ILbeta + IL10	94.5 93.5	94.5 93.3	94.5 93.8	94.5 93.8	94.6 93.4	301.9 211.7
IL17A + IFNgamma	92.5 91.2	90.4 89.1	94.5 93.3	94.3 93.1	91.0 89.8	165.0 115.6

In each cell, the first value is referred to the apparent performance measures and the second, to the corrected performance measures by the level of optimism calculated using a bootstrap procedure.

Figures 3 (A,B,C) and 4 (A,B,C) show the ROC curves and calibration plots, including the bias-corrected measures, of the six cytokine-based models. The smoking-adjusted models based on IL1alpha and IL17A + IFNgamma were the best calibrated graphically, showing these predictors a linear effect on the outcome.

The IL1alpha model presented a bc-intercept of -0.022 and a bc-slope of 0.933, and the IL17A + IFNgamma model, -0.013 and 0.885, respectively. The values of the Hosmer-Lemeshow test were non-significant (p= 0.504 and 0.522, respectively).

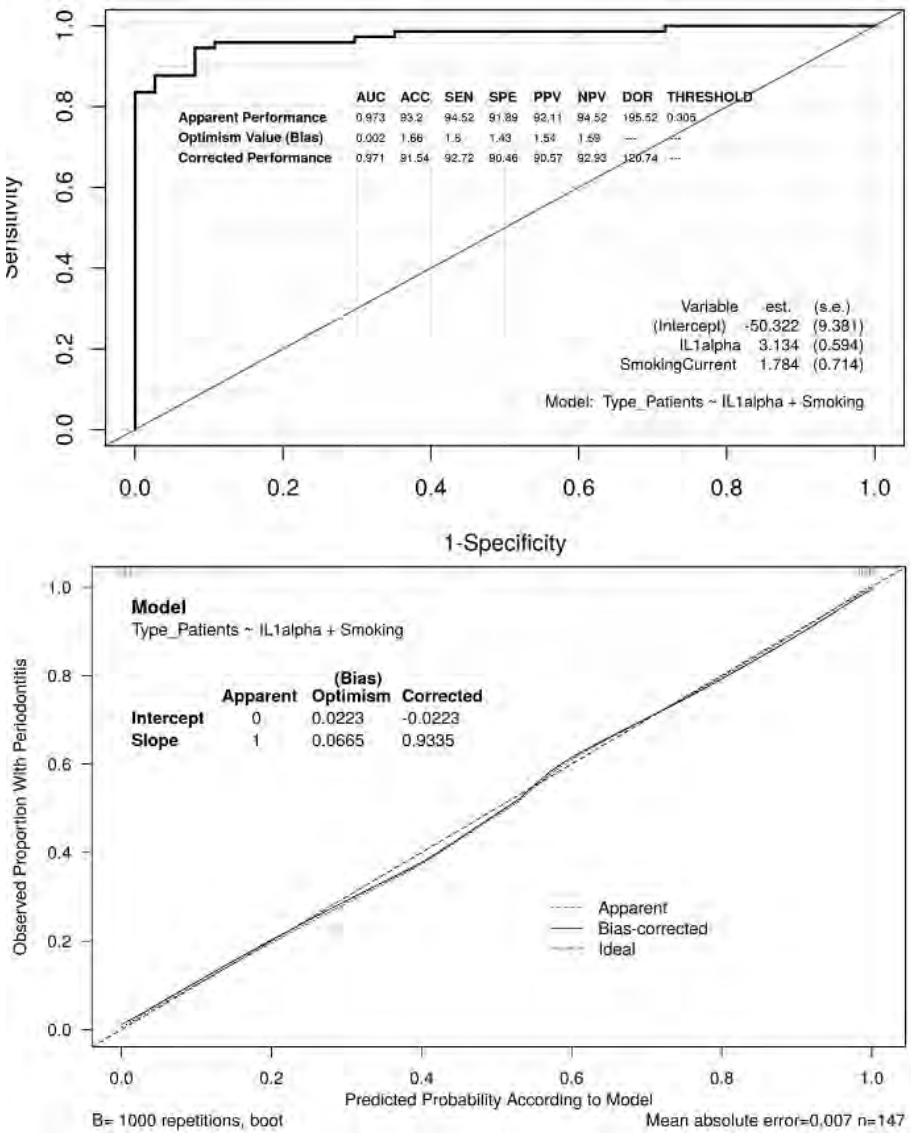

Figure 3A. ROC curves and calibration plots for IL1alpha model. There are included apparent and bias-corrected measures by bootstrapping. In the calibration plots, the predicted probability of the model is represented on the x-axis, and the observed proportion of chronic periodontitis is represented on the y-axis. The 45° line indicates perfect congruity between the predicted probability and the observed proportion of chronic periodontitis.

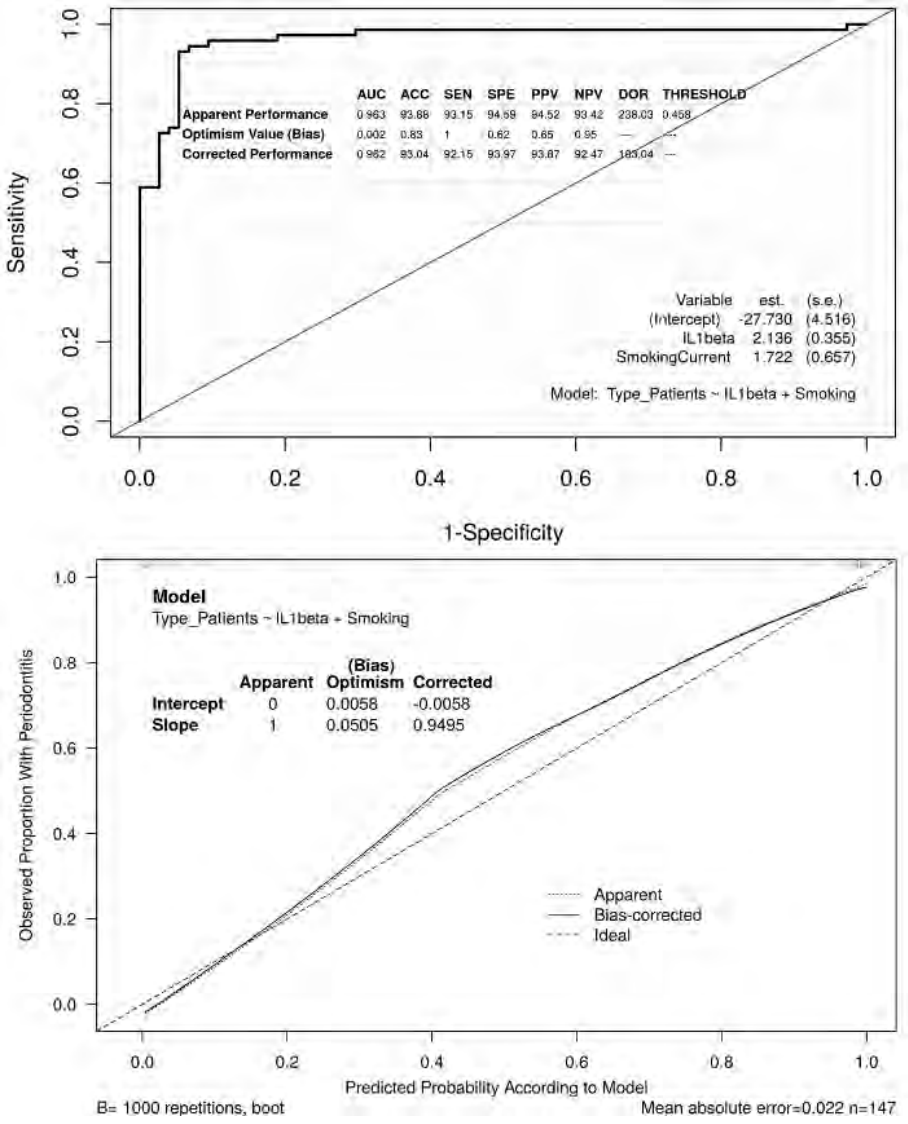

Figure 3B. ROC curves and calibration plots for IL1beta model. There are included apparent and bias-corrected measures by bootstrapping. In the calibration plots, the predicted probability of the model is represented on the x-axis, and the observed proportion of chronic periodontitis is represented on the y-axis. The 45° line indicates perfect congruity between the predicted probability and the observed proportion of chronic periodontitis.

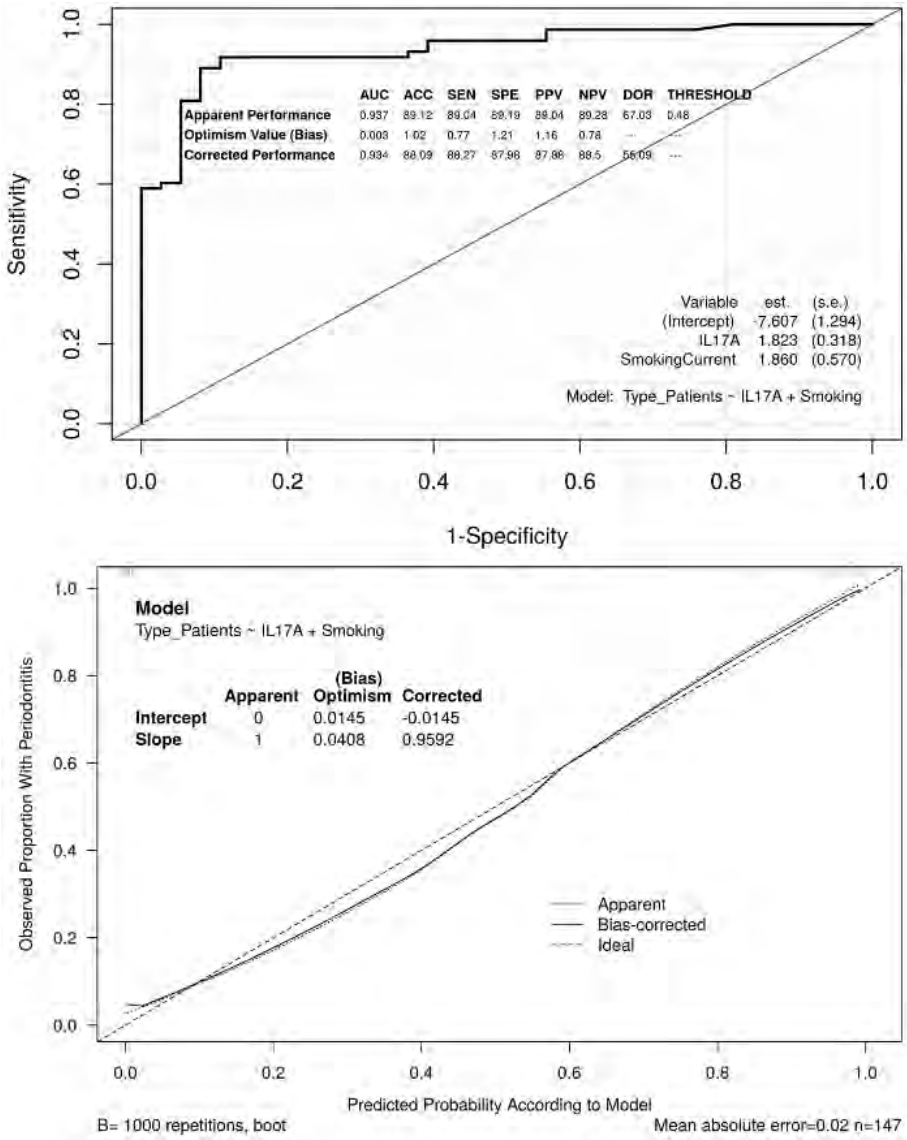

Figure 3C. ROC curves and calibration plots for IL17A model. There are included apparent and bias-corrected measures by bootstrapping. In the calibration plots, the predicted probability of the model is represented on the x-axis, and the observed proportion of chronic periodontitis is represented on the y-axis. The 45° line indicates perfect congruity between the predicted probability and the observed proportion of chronic periodontitis.

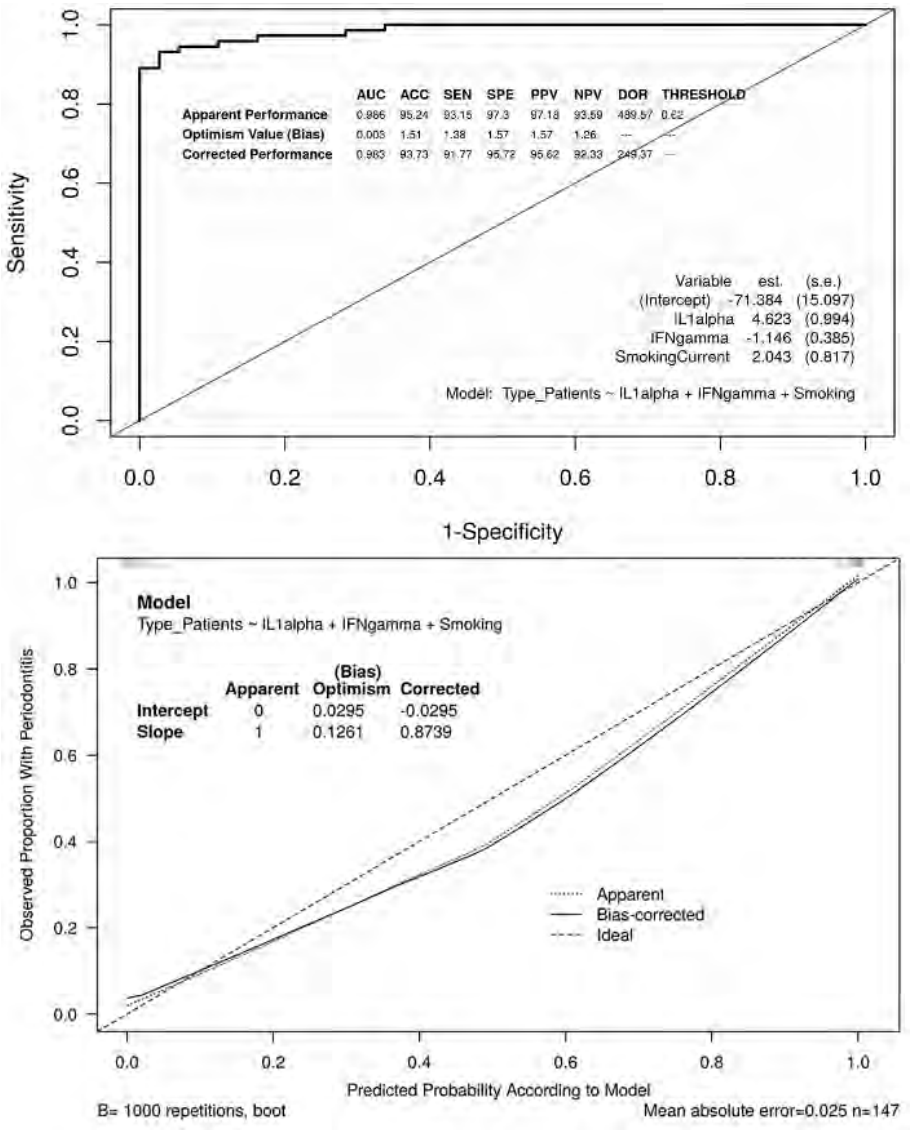

Figure 4A. ROC curves and calibration plots of IL1alpha + IFNgamma model. There are included apparent and bias-corrected measures by bootstrapping. In the calibration plots, the predicted probability of the model is represented on the x-axis, and the observed proportion of chronic periodontitis is represented on the y-axis. The 45° line indicates perfect congruity between the predicted probability and the observed proportion of chronic periodontitis.

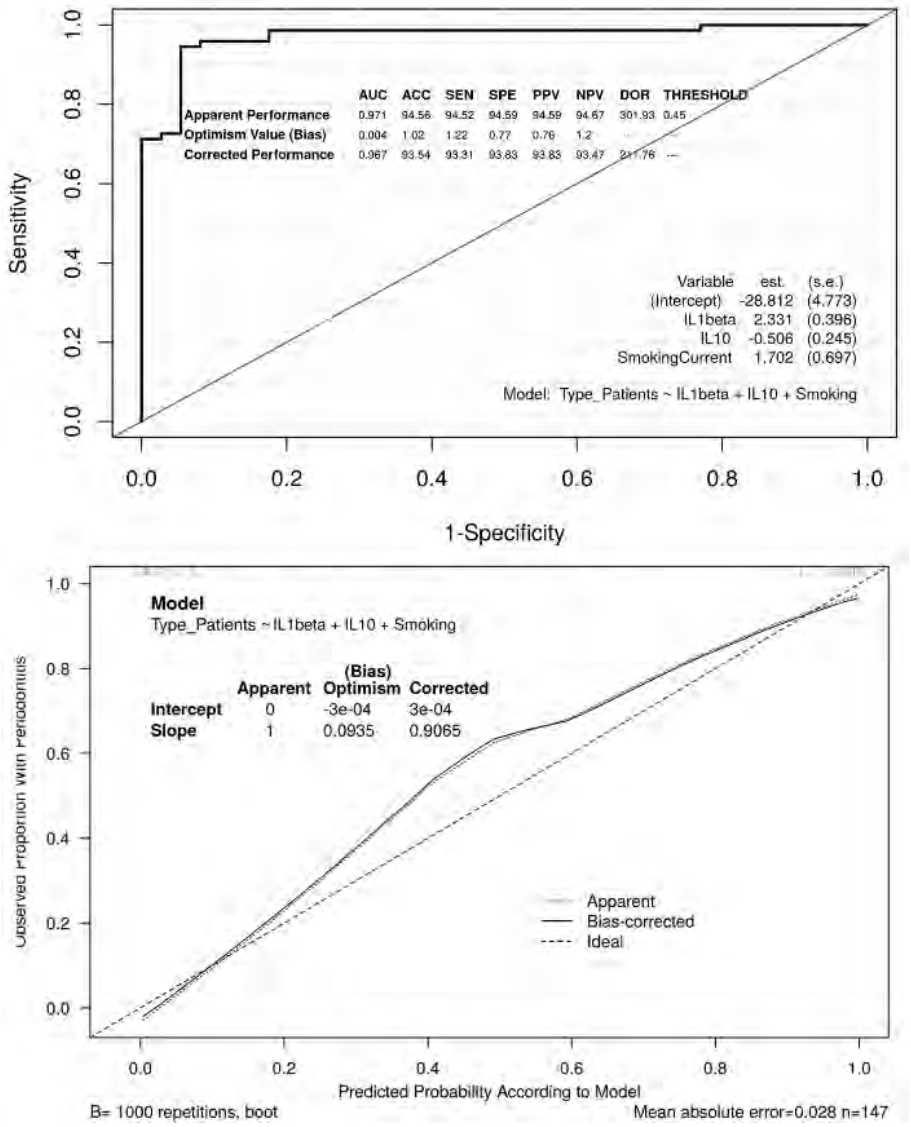

Figure 4B. ROC curves and calibration plots of IL1beta + IL10 model. There are included apparent and bias-corrected measures by bootstrapping. In the calibration plots, the predicted probability of the model is represented on the x-axis, and the observed proportion of chronic periodontitis is represented on the y-axis. The 45° line indicates perfect congruity between the predicted probability and the observed proportion of chronic periodontitis.

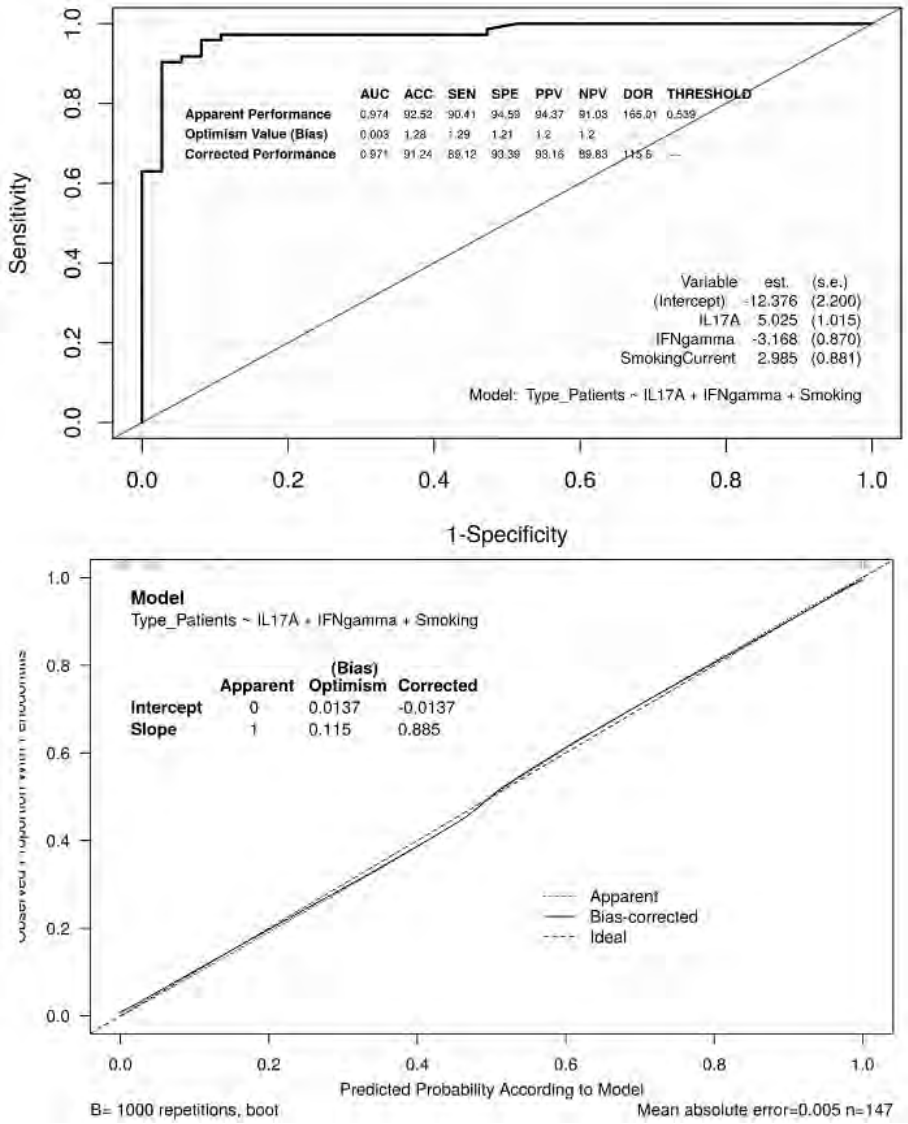

Figure 4C. ROC curves and calibration plots of IL17A + IFNgamma model. There are included apparent and bias-corrected measures by bootstrapping. In the calibration plots, the predicted probability of the model is represented on the x-axis, and the observed proportion of chronic periodontitis is represented on the y-axis. The 45° line indicates perfect congruity between the predicted probability and the observed proportion of chronic periodontitis.

Figures 5 (A,B,C) and 6 (A,B,C) show the diagnostic nomograms derived from the six cytokine-based models. As it has been commented upon previously, the discrimination and classification performance values of these nomograms were very high, indicating their outstanding accuracy.

According to the calibration parameters, especially the nomogram based on the IL1alpha and IL17 + IFNgamma were very reliable, because these showed very good correspondence between the actual outcomes and the predicted probabilities of having chronic periodontitis. In general, in three nomograms of two cytokines, higher levels of pro-inflammatory cytokines were associated with an increased probability of having chronic periodontitis, as did being a smoker. On the contrary, IFNgamma and IL10 had an opposite function to the pro-inflammatory ones, as higher levels of these mediators were associated with a reduced probability of having periodontitis.

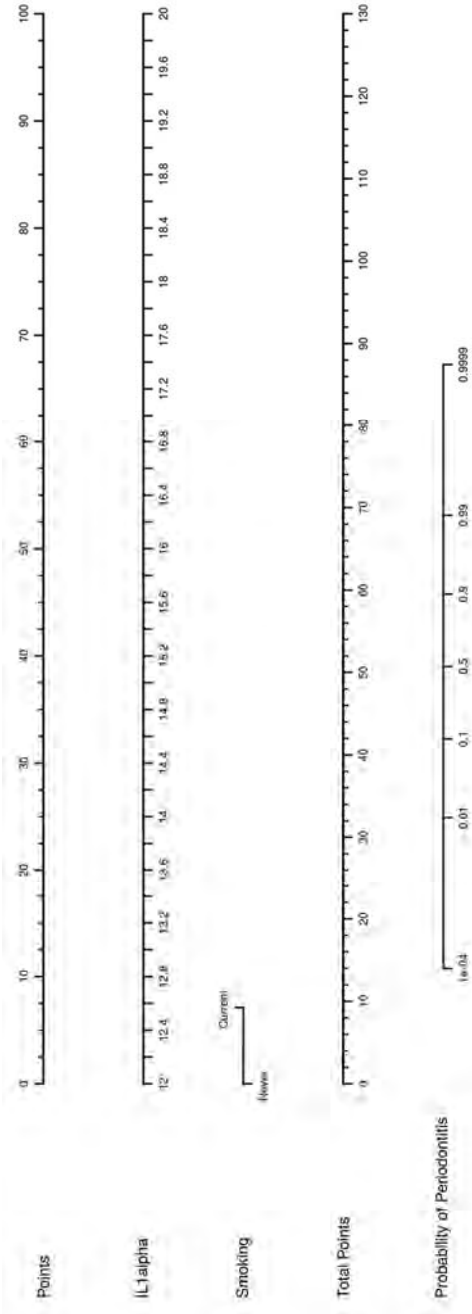

Figure 5A. Nomograms based on the IL1alpha model predicting the probability of chronic periodontitis. The probability of chronic periodontitis is calculated by drawing a line to the point on the axis for each of the following variables: IL1alpha and "smoking". The points for each variable are summed and located on the total point line. Next, a vertical line is projected from the total point line to the predicted probability bottom scale to obtain the individual probability of chronic periodontitis.

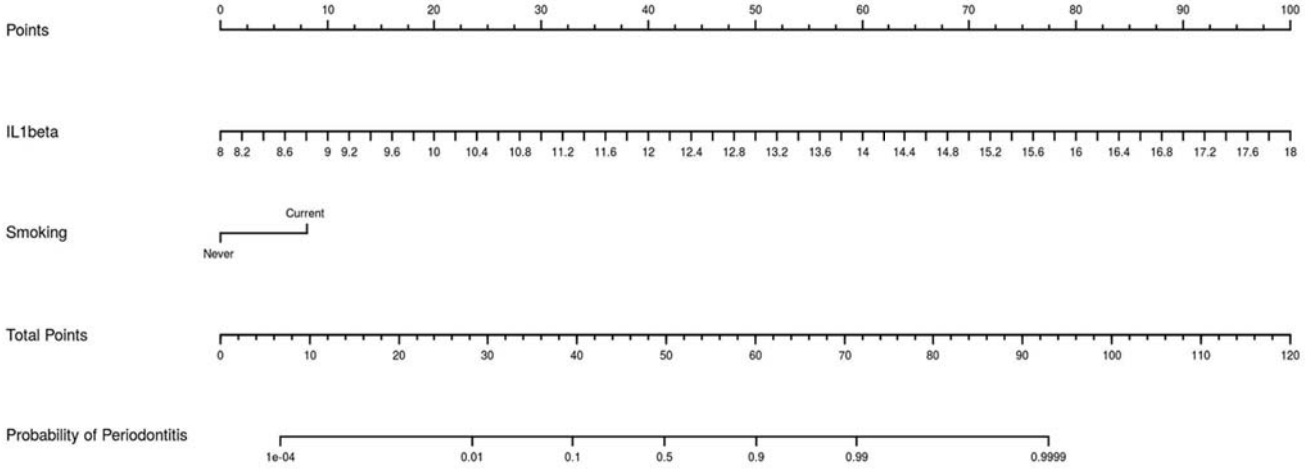

Figure 5B. Nomograms based on the IL1beta model predicting the probability of chronic periodontitis. The probability of chronic periodontitis is calculated by drawing a line to the point on the axis for each of the following variables: IL1beta and “smoking”. The points for each variable are summed and located on the total point line. Next, a vertical line is projected from the total point line to the predicted probability bottom scale to obtain the individual probability of chronic periodontitis.

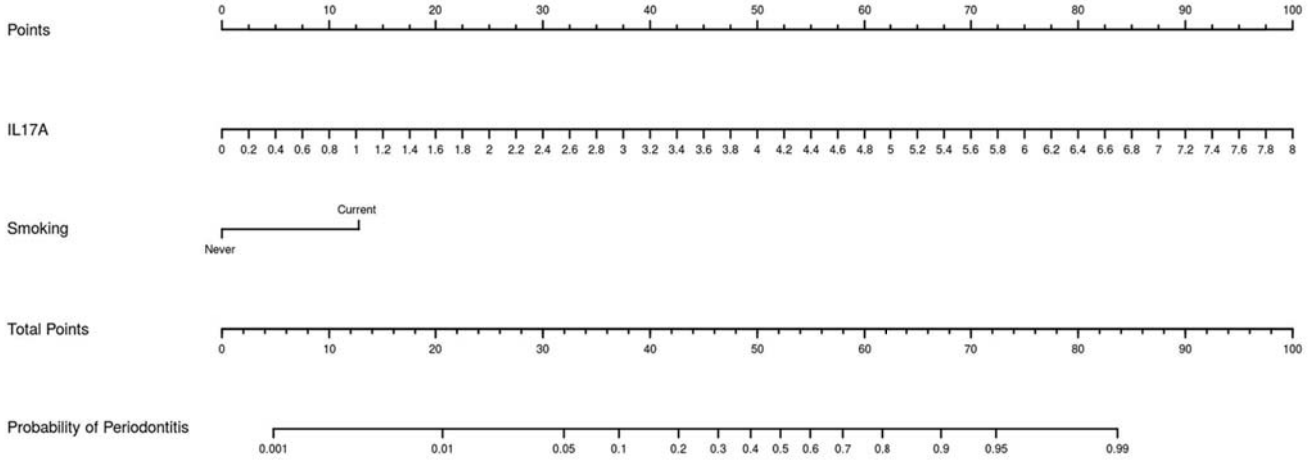

Figure 5C. Nomograms based on the IL17A model predicting the probability of chronic periodontitis. The probability of chronic periodontitis is calculated by drawing a line to the point on the axis for each of the following variables: IL17A and "smoking". The points for each variable are summed and located on the total point line. Next, a vertical line is projected from the total point line to the predicted probability bottom scale to obtain the individual probability of chronic periodontitis.

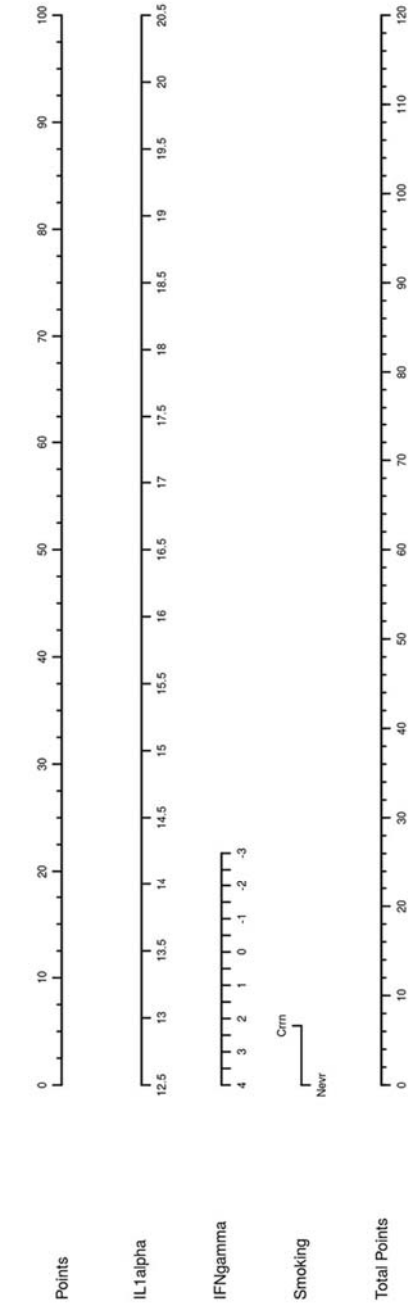

Figure 6A. Nomograms based on the IL1alpha + IFNgamma model predicting the probability of chronic periodontitis. The probability of chronic periodontitis is calculated by drawing a line to the point on the axis for each of the following variables: IL1alpha, IFNgamma and "smoking". The points for each variable are summed and located on the total point line. Next, a vertical line is projected from the total point line to the predicted probability bottom scale to obtain the individual probability of chronic periodontitis.

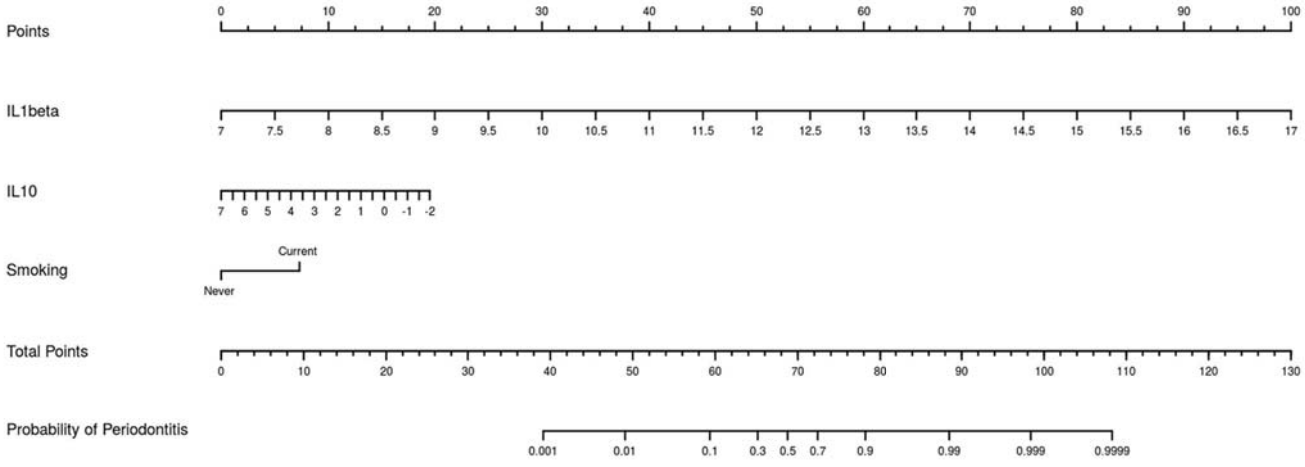

Figure 6B. Nomograms based on the IL1beta + IL10 model predicting the probability of chronic periodontitis. The probability of chronic periodontitis is calculated by drawing a line to the point on the axis for each of the following variables: IL1beta, IL10 and "smoking". The points for each variable are summed and located on the total point line. Next, a vertical line is projected from the total point line to the predicted probability bottom scale to obtain the individual probability of chronic periodontitis.

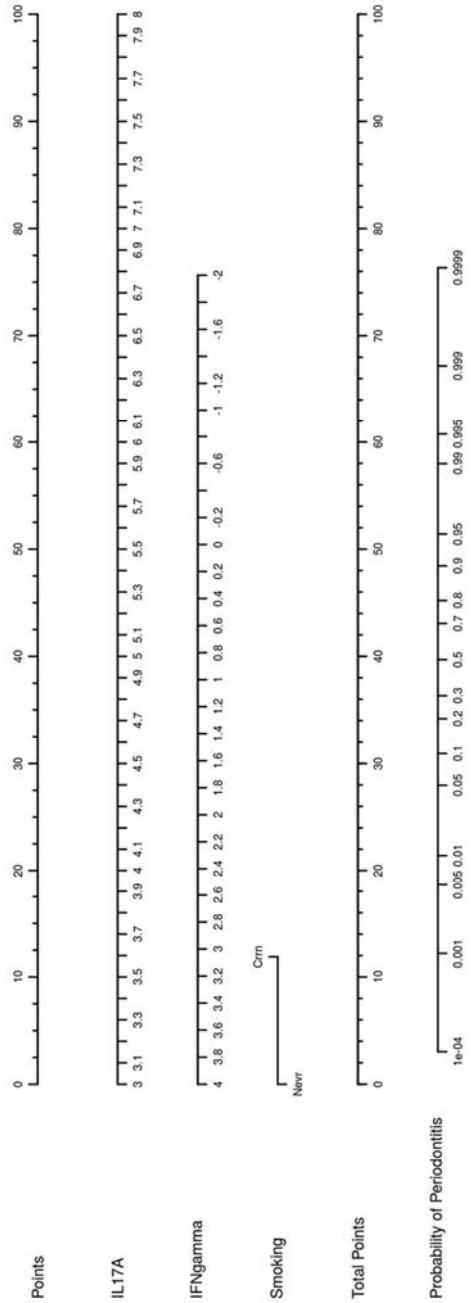

Figure 6C. Nomograms based on the IL17A + IFNgamma model predicting the probability of chronic periodontitis. The probability of chronic periodontitis is calculated by drawing a line to the point on the axis for each of the following variables: IL17A, IFNgamma and "smoking". The points for each variable are summed and located on the total point line. Next, a vertical line is projected from the total point line to the predicted probability bottom scale to obtain the individual probability of chronic periodontitis.

3.5. Discussion

The importance of cytokines in the pathogenesis of periodontal disease has been demonstrated in different stages. Not only do they act as initiators and regulators of the innate and adaptive immune response, but they also mediate the tissue damage that leads to a loss of function and clinical disease [15,21,49]. Specifically, cytokines such as GMCSF, IL1beta, IL6, IL17 and TNFalpha are among the more critical pro-inflammatory mediators when it comes to stimulating osteoclast activation [18,19]. On the other hand, anti-inflammatory cytokines such as IL2, IL4, IL5, IL10 and IL13 play a significant role in the regulation of T-cell subsets that act at several levels, with some of them having an inhibitory effect on osteoclastogenesis (IL13 and IFNgamma) [18,19].

3.5.1. Methodological Issues of the Published Papers

Numerous papers published in the literature have reported the quantification of cytokines in GCF, confirming that there is a distinct cytokine profile for patients with chronic periodontitis [18,26]. In a recently published meta-analysis study, however, Stadler et al. [26] demonstrated that many of these studies were underpowered (using a small number of subjects and limited assays) and based on a classical reductionist approach because they were focused on just a few cytokines at a time. Although it is generally accepted that the immunopathogenesis of periodontitis is driven by complex and dynamic networks of cytokine interactions [15], very few authors have evaluated the simultaneous presence of more than ten cytokines in GCF [27–29].

In the present study, we used a ‘‘knowledge-based’’ approach and included pro-inflammatory and anti-inflammatory cytokines to construct our panel of 16 biomarkers, selecting those with significant evidence of their role in the pathophysiology of chronic periodontitis. By selecting different classes of cytokines, we aimed to reduce the chances of redundancy among these mediators, potentially improving the diagnostic properties of the multi-cytokine models.

Unlike the position observed in numerous studies [26], the pooled GCF sample collected from 20 subgingival sites of each patient allowed us to obtain a representative patient-level descriptor of the whole-mouth periodontal inflammatory response at certain times [50]. This premise can be related to a patient-level periodontitis status and possibly a patient-level disease risk. In the present study, we used a multiplex bead immunoassay for the simultaneous analysis of 16 cytokines in the GCF samples. Despite being a technique applied in less than 20% of the series [26], these immunoassays provide a sensitivity that is equal to or better than the conventional enzyme-linked immunosorbent assays and have the ability to measure multiple mediators simultaneously (up to 100 different analytes) in volumes as small as 50 µl or less. In contrast, such volumes would only be sufficient for the analysis of a single cytokine using the ELISA technique. Furthermore, the costs per analyte are relatively low when many of them are measured, and processing times are short [51,52]. Like the ELISA or any other immunoassay, the sensitivity and specificity of multiplexed microsphere assays depend on the use of high affinity, high specificity antibodies [52].

The term 'biomarker' refers to a biologic compound which can be quantified and analysed to serve as a biological indicator of physiological or pathogenic processes, environmental exposure and response to therapeutic interventions [53]. Oral fluids such as GCF and saliva, and even blood, have emerged as potential diagnostic tools for the detection of the biomarkers associated with periodontitis [54–56]. However, in terms of the cytokines in GFC, the majority of studies are designed to be cross-sectional, in which the cytokine levels in healthy volunteers and periodontal patients are compared for testing, whether or not a particular mediator is increased/decreased at the individual level [21,26]. There is, therefore, no evidence in the literature on the development of GCF cytokine-based predictive models for the diagnosis or prognosis of periodontitis using appropriate multivariate predictive modelling techniques [30]. These techniques are useful tools with which to evaluate not just a single biomarker, but also a panel of biomarkers, which increases their diagnostic and prognostic power [30]. To the best of the authors' knowledge, this is the first study in which diagnostic predictive models based on 16 cytokine levels in GCF

have been evaluated and validated internally by multivariate predictive modelling techniques.

3.5.2. Comparison of GCF Cytokine Levels between Periodontally Healthy Individuals and Patients with Chronic Periodontitis

In order to interpret a large number of different results published in the literature on the GCF cytokine levels in chronic periodontitis, Stadler et al. [26] recently conducted a meta-analysis on the subject. According to this, IL1beta is the most studied cytokine in the pathogenesis of chronic periodontitis, while very few papers have focused on the role of anti-inflammatory cytokines (with IL4 and IL10 being the most researched) [26]. These authors found evidence of significant differences between chronic periodontitis and periodontal health for only a few pro-inflammatory cytokines (IL1beta, IL6 and IL17, which showed higher levels for periodontitis than health). This first conclusion is corroborated by the results of the present study, although we also detected significantly elevated levels of other pro-inflammatory cytokines such as GMCSF, IL1alpha, IL12p40 and TNFalpha. In our series, IL1alpha and IL1beta were the most important pro-inflammatory cytokines in terms of increased concentration associated with the disease, followed by IL6 and GMCSF, and IL17A and TNFalpha.

Equally, in the Stadler's meta-analysis [26], evidence of significant differences between chronic periodontitis and periodontal health was only found for a few anti-inflammatory cytokines (IFNgamma and IL4, which showed higher levels for health than periodontitis). Curiously, however, our results were contrary to those reported by these authors, as four anti-inflammatory cytokines (IFNgamma, IL2, IL3 and IL4) showed significantly higher concentrations in chronic periodontitis than in health. These increases were lower than those detected for the pro-inflammatory cytokines (except for IL3). These discrepancies could be due to methodological differences detected in the studies about the number of subgingival sites sampled and GCF levels obtained, which

in many series could be insufficient to determine the levels of anti-inflammatory cytokines.

3.5.3. Multivariate Predictive Modelling of GCF Cytokine Levels: Selection of the Best Models and the Development of Nomograms

In periodontology, traditional clinical criteria are often inadequate for: 1) determining sites of active disease; 2) measuring the degree of susceptibility to future progression; 3) monitoring quantitatively the response to therapy. In this sense, there is a need for research on innovative diagnostic tests based on biomarkers that focus on the early recognition of the microbial challenge to the host, detecting real-time changes in the periodontium [15,17]. On the other hand, the role of inflammation appears to be the common denominator between periodontitis and some of the systemic diseases and conditions mentioned in the Introduction to this Thesis. These circumstances emphasise the importance of utilising oral fluid diagnostic methods for monitoring periodontal diseases [15]. However, to date, limited research has been completed on the use of GCF as a diagnostic measure of periodontal disease [57].

As an ideal diagnostic test should have predictive accuracy values approaching 100% [18], in the present series, we surprisingly obtained several GCF cytokine-based models (six models) formed by one to two cytokines. These models were supported by the well-known biological role of the cytokines involved, could discriminate chronic periodontitis of >0.93 and were statistically validated models. Consequently, they were considered “outstanding predictive models” [40] and demonstrated that cytokines could be excellent biomarkers when it comes to distinguishing patients with chronic periodontitis from periodontally healthy individuals.

There are, however, contrary opinions defending the notion that cytokines are not specific enough for predicting periodontitis, and their levels in the GCF may be affected by local or systemic factors such as smoking, alcohol consumption and stress [16,58]. It has been stated that

cytokine networks are complex, interactive, continuously changing, and have a redundant functionality, meaning that the interpretation of cytokine levels at one particular point in time is fraught with error [59]. In our opinion, however, these affirmations are questionable in the face of strong evidence on the predictive ability of certain cytokines found in the present study.

Regarding the influence of other variables, smoking is a well-established traditional risk factor for chronic periodontitis [60,61], and its influence in the levels of some cytokines in the GCF from periodontal patients has been highlighted by previous authors [28,62]. In the present series, we observed in the GCF cytokine-based models that smoking status increased by 15-20% the probability of having chronic periodontitis.

As there is no literature on the subject, our cytokine-based predictive models could not be compared to similar models, and we have had to consider predictive studies of periodontitis based on other biomarkers detected in the GCF [59,63]. It is highly unlikely that a single biomarker can be a stand-alone measure for predicting periodontitis activity [16], and several systemic conditions may affect the GCF levels of a single biomarker [64]. In the present study, assuming the key role developed by pro-inflammatory cytokines in the pathogenesis of chronic periodontitis [21], models were constructed by initially selecting one pro-inflammatory cytokine as a predictor variable. Surprisingly, the smoking-adjusted models formed by IL1alpha, IL1beta and IL17A showed a high discriminative power (bc-AUC>0.93), which resulted in corrected percentages of classification measures >87%. Of these pro-inflammatory cytokines, IL1beta was the one that presented the best predictive parameters (corrected classification measures >92%), followed by IL1alpha (corrected classification measures >90%) and IL17A (corrected classification measures >87%). It is very interesting to note the high predictive ability of IL17A, although this cytokine did not show an increase in levels associated with the disease especially high compared to that observed in other pro-inflammatory cytokines. These findings on the outstanding

predictive accuracy of these pro-inflammatory cytokines in the GCF have not been previously described in the literature.

However, a combined analysis of different valuable host-responses is required to identify the set of biomarkers with the most favourable combination of sensitivity, specificity and reproducibility [16,57]. On the other hand, it is advisable to search for smaller multi-biomarker models, which are easier to apply in clinical practice than larger versions [30,65]. In line with the GCF multi-biomarker predictive models for having peri-implantitis described by Zani et al. [66], we analysed the two-variable models that combined IL1alpha, IL1beta and IL17A with anti-inflammatory cytokines. The purpose was to test if the predictive capacity of these pro-inflammatory cytokines can be increased by the incorporation of cytokines with anti-inflammatory effects. Three smoking-adjusted models were found that fulfilled this premise: IL1alpha + IFNgamma, IL1beta + IL10 and IL17A + IFNgamma (bc-AUC>0.96; corrected classification measures >89%). To the best of our knowledge, these results are the first evidence on the high predictive ability of models based on a pro-inflammatory cytokine and another anti-inflammatory to distinguish a patient with chronic periodontitis from one with periodontal health.

According to TRIPOD [30], it was very interesting to test how, although IL10 alone showed non-significant high levels in chronic periodontitis, this acquired an important value within the two-biomarker model, increasing the discriminative capacity of the IL1beta. This corroborates, together with the finding previously commented on IL17A, the importance of designing future studies focused on predictive analysis that are properly powered. In these studies, a large number of periodontitis-related mediators should be evaluated using appropriate multivariate statistical techniques [21,26].

In the present series, the best predictive models according to their bc-ACC values were formed by IL1alpha + IFNgamma (bc-AUC>0.98, bc-ACC>93% and other corrected classification measures >91%) and IL1beta + IL10 (bc-AUC>0.96, bc-ACC>93% and other corrected classification measures >93%), followed by IL17A + IFN gamma (bc-

AUC>0.97, bc-ACC>0.91 and other corrected classification measures >89%). This accuracy in terms of predicting chronic periodontitis is much higher than that found in previous studies based on other biomarkers detected in GCF. As a consequence, the model recently validated by Kim et al. [63], which consisted of matrix metalloproteinases (MMP8, MMP9, and MMP13), had an AUC value of 0.84, with a sensitivity and specificity of 70% and 86%, respectively. When other demographic variables and risk factors (age, sex, income, smoking, drinking), as well as blood cytokine levels (IL6, IL8 and TNFalpha), were included in this model, the AUC value increased up to 0.86.

Nomograms are simplified representations of complicated statistical models, and their clinical value relates to the fact that they map the predicted probabilities into points on a scale from zero to 100 in a user-friendly graphical interface [46]. To our knowledge, this is the first study providing several nomograms based on GCF cytokine levels to predict the probability of having chronic periodontitis.

The use of only a few variables is desirable in nomograms to increase their utility in clinical practice [65]. It has been suggested that the proper calibration of a nomogram is more clinically useful than its discriminatory capacity [66]. Our nomograms, which are derived from the one- and two-cytokine models, fulfilled the requirements of discrimination. According to the calibration measures, especially the nomogram based on the IL1alpha and IL17A + IFNgamma were very reliable. If these tools were used in the field of clinical activity, the diagnosis of patients at risk of developing chronic periodontitis could be improved, leading to better, more cost-effective, methods of prevention and treatment of this disease.

On the other hand, Preshaw and Taylor, in an excellent review published in 2011 [21], concluded that cytokines interact and function within networks, but we do not yet understand how the imbalance of the networks relates to the clinical course of periodontitis. In this regard, we are providing evidence through the development of cytokine-based predictive models and their corresponding nomograms. Interestingly, in

the nomograms with more than one cytokine, unlike the position with IL1alpha, IL1beta, IL7A and smoking status, higher levels of IFNgamma and IL10 are associated with low scores. These findings reveal the level of the periodontitis-associated imbalance between these pro-inflammatory (potentiating role) and these anti-inflammatory cytokines (protective role) in terms of a particular probability of having periodontitis.

Our research has some limitations. The most significant weakness is that the prediction of the study's accuracy is only measured in the samples that generated the model equations. As a consequence, to evaluate the reproducibility of the models, we validated the prediction rule internally (calibration, discrimination and classification measures) using bootstrap methods on the original derivation dataset by sampling with replacements for 1000 iterations [48,67]; the results in this respect were quite optimal. Another limitation was the sample size. Although we are in a scenario of small data, we believe that the concordance between the results obtained and the biological knowledge of the subject indicate that we are on the right way.

The evaluation of these GCF cytokine-based predictive models and nomograms is a potential future research direction. Firstly, it would greatly benefit the strength of our study if the predictive accuracy of the predictive models derived from our series could be measured in an ''external'' or independent cohort of patients to verify whether our findings are universally applicable. Secondly, the potential prognostic value of these predictive models concerning clinical progression of the disease and the response to treatment in periodontal patients should be exploited in longitudinal studies, as should their potential predictive accuracy in saliva samples.

In conclusion, of the 16 evaluated cytokines, the GCF levels of the eight pro-inflammatory (GMCSF, IL1alpha, IL1beta, IL6, IL12p40, IL17A, IL17F and TNFalpha) and four anti-inflammatory cytokines (IFNgamma, IL2, IL3 and IL4) were significantly elevated in the patients with chronic periodontitis, with this increase in the concentrations being stronger in the pro-inflammatory cytokines. The

outstanding predictive accuracy of the resulting smoking-adjusted models showed that IL1alpha, IL1beta and IL17A in GCF are outstanding biomarkers for distinguishing systemically healthy patients with chronic periodontitis from periodontally healthy individuals. The predictive ability of these pro-inflammatory cytokines was increased by incorporating IFNgamma and IL10. In the nomograms developed herein, higher levels of these pro-inflammatory cytokines and being a smoker increased the probability of having chronic periodontitis (potentiating role), while IFNgamma and IL10 had the opposite function (protective role). The clinical implications of these predictive tools could include improved patient monitoring and the control of disease activity. However, additional evidence is needed to test the external validity of these GCF cytokine-based models for predicting chronic periodontitis and their value for the clinical use of proposed nomograms.

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OBJECTIVE 4

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Objective 4. Cytokine Thresholds in Gingival Crevicular Fluid with Potential Diagnosis of Chronic Periodontitis Differentiating by Smoking Status

4.1. ABSTRACT

Aim: To determine cytokine thresholds derived from predictive models for the diagnosis of chronic periodontitis, differentiating by smoking status.

Material and Methods: A total of 175 systemically healthy individuals, including seventy-five periodontally healthy controls and 75 subjects affected by chronic periodontitis were recruited. Sixteen mediators were measured in gingival crevicular fluid (GCF) using multiplexed bead immunoassays. The models were obtained using binary logistic regression, distinguishing between non-smokers and smokers. The area under the curve (AUC) and numerous classification measures were obtained. Model curves were constructed graphically, and the cytokine thresholds calculated for the values of maximum accuracy (ACC).

Results: There were three cytokine-based models and three cytokine ratio-based models, which provided bias-corrected values of AUC >0.91 and >0.83, respectively. These models were (cytokine thresholds in pg/ml for the median ACC using bootstrapping for smokers and non-smokers): IL1alpha (46099 and 65644); IL1beta (4732 and 5827); IL17A (11.03 and 17.13); IL1alpha/IL2 (4210 and 7118); IL1beta/IL2 (260 and 628); and IL17A/IL2 (0.810 and 1.919).

Conclusions: IL1alpha, IL1beta and IL17A, and their ratios with IL2 appeared to be excellent diagnostic biomarkers in GCF for distinguishing systemically healthy subjects with chronic periodontitis from periodontally healthy individuals. Cytokine thresholds in GCF with diagnostic potential are defined, showing that smokers have lower threshold values than non-smokers. Potential applications of these models in the clinical practice are discussed.

4.1.1. Keywords

Chronic periodontitis, smoking, gingival crevicular fluid, cytokines, immunoassay, area under curve, thresholds, sensitivity, specificity.

4.1.2. Declaration of Conflict of Interest

The doctoral candidate and the rest of the authors of the present study declare that they have no conflict of interest concerning the objectives proposed in this chapter.

4.1.3. Funding

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4.2. INTRODUCTION

Periodontitis is a public health problem, as it is highly prevalent and causes disability and social inequality [1]. In 2010, severe periodontitis was estimated to be the sixth most prevalent disease globally, affecting 743 million people worldwide [2]. Periodontitis is currently being connected bidirectionally to the pathogenesis of various conditions and systemic diseases of high morbi-mortality such as diabetes, coronary heart disease, metabolic syndrome, chronic respiratory diseases, rheumatoid arthritis, cancer, obstetric complications or cognitive impairment, among others [3,4].

It is now widely accepted that, although the initiating factor is a polymicrobial dysbiosis [5], the pathogenesis of periodontitis is driven by the development of a chronic inflammatory host immune response [6,7]. The nature and extent of this response are fundamental determinants of the susceptibility to and progression of periodontitis [6,7].

Cytokines are soluble protein 'messenger' molecules produced by a variety of cells that transmit signals to other cells [8]. Cytokines play a crucial role in initiating and sustaining the inflammatory immune response by stimulating the production of secondary mediators. These mediators, in turn, evoke a cascade of events that amplify the inflammatory response and induce the production of enzymes that are responsible for the degradation of connective tissue and osteoclastic bone resorption [9].

Cytokines interact and function within a complex and dynamic network of interactions, rather than being dominated by the action of individual cytokines [8]. An imbalance between the pro-inflammatory and anti-inflammatory cytokines derived from Th1, Th2, Th17 and Treg lymphocyte subpopulations is suggested as being responsible for periodontal breakdown through cellular and humoral hyper-immune responses [10]. However, the reductionist approach is predominant *in vivo* research, as very few authors have analysed the simultaneous presence of more than ten cytokines [11-13], or more than four cytokine

ratios, in gingival crevicular fluid (GCF) from periodontal patients [14,15]. Accordingly, more evidence is required from multiple cytokine analyses to increase what is understood of this complex and dynamic network [8].

On the other hand, the detection of biomarkers in GCF for predicting the early onset of periodontitis or evaluating the untreated or treated disease activity is a crucial challenge in Periodontology [16-18]. There is, however, limited literature on the development and validation of predictive models based on GCF cytokine levels for the diagnosis or prognosis of periodontitis [19,20].

Accordingly, the objectives of this cross-sectional study were:

1) To compare the levels of 16 cytokines detected in GCF, as well as multiple cytokine ratios obtained from them, in systemically healthy individuals with periodontal health and patients with chronic periodontitis.

2) To determine the diagnostic value thresholds derived from cytokine-based and cytokine ratio-based models in non-smokers and smokers, selecting those models with a high discriminatory capacity to distinguish between periodontal patients and periodontally healthy controls.

3) To validate cytokine-based and cytokine ratio-based models internally using bootstrapping techniques, describing their diagnostic thresholds, as well as apparent and corrected measures of discrimination and classification.

4.3. Materials and Methods

4.3.1. Selection of Study Groups

A sample of 150 systemically healthy participants was recruited (Table 1) among 250 consecutive patients from the general population who were referred to the School of Medicine and Dentistry (Universidade de Santiago de Compostela, Spain) for an evaluation of their oral health status between 2013 and 2015. This sample consisted of all the cases (patients with the target condition, that is, 75 subjects affected by moderate to severe generalised chronic periodontitis -perio group-) and a random sample of the periodontally healthy controls (75 subjects, -control group-). Patients were selected if they fulfilled the inclusion criteria, which are detailed in the Objective 3.

One previously calibrated, experienced dentist performed all the periodontal examinations. The probing pocket depth (PPD) and the clinical attachment level (CAL) were recorded on all teeth at six sites per tooth using a PCP-UNC 15 probe. The bleeding on probing (BOP) and the bacterial plaque level (BPL) data were recorded for the full mouth on a binary scale (presence/absence) on six sites per tooth. Standardised radiographs of all teeth were obtained to assess the alveolar bone status.

The presence of periodontal health or moderate to severe generalised chronic periodontitis was established according to the clinical/radiographic information, applying previously published criteria [21,22]. Smoking histories were obtained using a questionnaire, with information collected on smoking status (never, past or current, the number of months of smoking and the number of cigarettes/day). All the answers were reviewed with the subject by a member of the study team.

This study was conducted according to the principles outlined in the Declaration of Helsinki (as revised in 2000) on experimentation involving human beings [23]. Patients who agreed to participate in the research provided written informed consent. The study's protocol was approved by the Clinical Research Ethics Committee of Galicia

(number 2015/006; Appendix S1). The TRIPOD and STARD guidelines on studies on diagnostic accuracy were considered; the TRIPOD checklist was completed (Appendix S2) [24].

4.3.2. Gingival Crevicular Fluid Sampling

The GCF collection took place one week after the initial examination, and the samples were obtained at the same time of day (in the afternoon, approximately 5-7 hours after tooth brushing). A paper strip (Periopaper, Amityville, NY, USA) was inserted into the gingival sulcus or periodontal pocket for 30 seconds, using a GCF collection protocol previously described [25]. GCF samples from the controls and periodontal patients were collected and pooled from 20 non-adjacent proximal sites. In the first case, samples were taken from subgingival sites from teeth in quadrants 1 and 3, and in the second case from sites from the most in-depth PPD in each quadrant.

Strips from each subject were inserted into labelled tubes with 300 ml of 0.01M PBS (pH=7.2) and a protease inhibitor (Complete Mini, protease inhibitor cocktail tablets, Roche Applied Science, Indianapolis, IN, USA). To ensure sample collection, the GCF volume was determined based on measurements of weighing the tubes and strips before and after sampling using a high-sensitivity scale [26] (readability of 0.01 mg; Explorer Semi Micro Ex125M, OHAUS, Greifensee, Switzerland). All the samples collected had volumes of GCF ≥10 µl. After obtaining the supernatant, the GCF samples were frozen at -80ºC until further biochemical analysis.

4.3.3. Quantification of Cytokines in Gingival Crevicular Fluid Using Multiplexed Bead Immunoassays

GCF cytokine levels were determined using the human cytokine 16-plex Procarta immunoassay (Affymetrix, Inc., Santa Clara, CA, USA). Sixteen mediators were measured: granulocyte-macrophage colony-stimulating factor -GMCSF-; IFNgamma; IL1alpha; IL1beta; IL2; IL3; IL4; IL5; IL6; IL10; IL12p40; IL12p70; IL13; IL17A; IL17F; and TNFalpha. The concentration range for each biomarker analysed is described in Objective 3.

A single investigator blinded to the clinical data performed the experimental analyses of the GCF cytokine quantification. The assays were performed in 96-well filter plates following the manufacturer's instructions and applying an analysis protocol described previously [25]. The GCF samples were quantified using the Luminex 100™ instrument (Luminex Corporation, Austin, Texas, USA), and all of them were run in duplicate. The concentrations of the unknown samples were estimated from the standard curve using a 5PL algorithm, and the Luminex IS 2.3 and xPONENT 3.1 software packages (Luminex Software, Inc.). Values were expressed as pg/ml, adjusting for the dilution factor. Samples below the detection limit (DL) of the assay were recorded as DL/2 [27], while those above the upper limit of quantification of the standard curves were assigned the highest value of the curve.

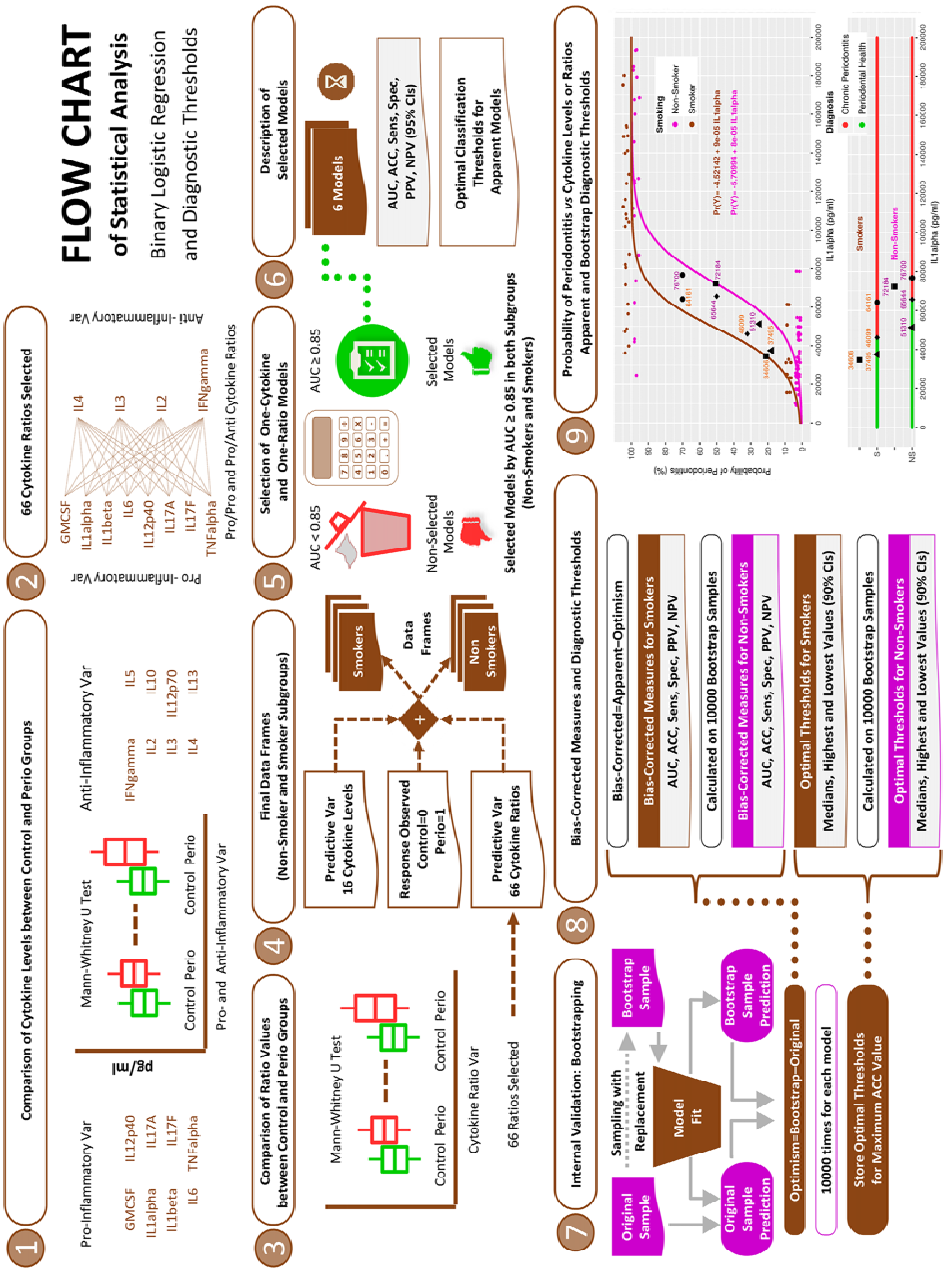

Figure 1. Flow chart of the statistical analysis: binary logistic regression and diagnostic thresholds.

4.3.4. Statistical Analysis

The statistical analyses were performed using the R software (version 3.4.3) [28]. It is available as Free Software under the terms of the Free Software Foundation's GNU General Public License in source code form. After applying the Shapiro-Wilks test and verifying the non-normal distribution of almost all the clinical variables, the Mann-Whitney U test was used to compare the quantitative variables between the perio and control groups. The Fisher's exact test was used to assess the association of the qualitative variables between both study groups. The significance level applied was a p value <0.05.

4.3.4.1. Comparison of GCF Cytokine Levels and Cytokine Ratio Values in Periodontally Healthy Individuals and Patients with Chronic Periodontitis

After verifying the non-normal distribution of variables using the Shapiro-Wilks test, the Mann-Whitney U test was used to compare the cytokine levels and cytokine ratios in the control and perio groups. The significance levels applied were adjusted by the Benjamini-Hochberg correction [29], with p values $\leq 1 \times 10^{-3}$ and $< 1 \times 10^{-5}$, respectively. A total of 66 cytokine ratios were evaluated, taking into account exclusively those cytokines that showed significant levels in the periodontal patients compared to the controls (Figure 1).

4.3.4.2. Predictive Modelling of Chronic Periodontitis Based on Cytokine Levels and Cytokine Ratios: Model Selection; Discrimination and Classification Measures; Determination of Diagnostic Thresholds; and Internal Validation

To obtain specific diagnostic thresholds differentiating by smoking status, we decided to develop different models for non-smokers (N= 93; 61 controls and 32 periodontal patients) and smokers (N= 54; 13 controls and 41 periodontal patients). In the calculation of the sample size, estimating a pre-determined AUC value of 0.850 with a given marginal error of 0.1 and 95% confidence level, the required sample size was around 38 subjects in each clinical condition, both non-smokers and smokers [30].

Models were obtained by binary logistic regression, selecting one cytokine or cytokine ratio as a predictor variable, which was treated in its original scale.

The statistical criterion applied for the model selection was the ability of each cytokine- or cytokine ratio-based model to determine the presence of chronic periodontitis using the value of the area under the curve (AUC) [31]. The AUC values and their corresponding 95% confidence intervals (CIs) obtained by bootstrapping were calculated using the pROC package (version 1.10.0) [32]. Only those models that presented an apparent AUC ≥0.85 in both types of model for smokers and non-smokers were selected [33].

The best cut-off value or optimal classification threshold for each model was defined as that which provides the maximum percentage of correct predictions (accuracy, ACC), and was calculated using the PresenceAbsence package (version 1.1.9) [34]. By setting this optimal value, various classification measures such as the ACC, the sensitivity (SENS), the specificity (SPEC), the positive predictive value (PPV), and the negative predictive value (NPV), as well as their corresponding 95% CIs acquired by bootstrapping, were obtained using the pROC package [32]. The respective cytokine levels or cytokine ratios were calculated for all the periodontitis probability values of each model, and the model curves were constructed graphically using the ggplot package (version 2.2.1) [35].

Regarding internal validation, bootstrapping was used to test for possible overfitting by determining the optimism values on the discrimination and classification measures. The bootstrap analysis was replicated on 10,000 random samples of the same sample size, drawn with replacements from the original sample [36,37]. Bias-corrected (bc) AUC and all other classification measures (bc-sensitivity, bc-specificity, bc-PPV, bc-NPV) were calculated as their corresponding apparent measures derived from the entire original sample minus optimism [36,37]. This technique was also used to define the cytokine thresholds for the median ACC values derived from 10,000 samples

from each model selected, as well as the thresholds for the 90% CIs of the ACC values (Figure 1).

4.4. RESULTS

Table 1. Age, gender, smoking habit and clinical characteristics associated with periodontal status in the control and perio groups.

CLINICAL PARAMETERS	STUDY GROUPS		
	Control group (n=74)	**Perio group (n=73)**	***P* Value**
Age (years)	45.65 (12.37)	51.12 (10.01)	0.005
Gender			
Male	31	31	NS
Female	43	42	
No. of teeth	26.72 (3.25)	25.55 (4.00)	NS
Full mouth			
BPL (%)	26.41 (18.66)	53.08 (26.77)	<0.001
BOP (%)	15.05 (6.61)	51.12 (20.07)	<0.001
PPD (mm)	2.11 (0.27)	3.49 (0.65)	<0.001
CAL (mm)	2.36 (0.46)	4.25 (1.12)	<0.001
Sampled sites			
BOP (%)	10.11 (10.24)	66.97 (23.93)	<0.001
PPD (mm)	2.23 (0.22)	5.65 (0.89)	<0.001
CAL (mm)	2.31 (0.27)	6.05 (1.12)	<0.001
Smoking habit[1]			
Non-smokers	61	32	<0.001
Smokers	13	41	
Cigarettes/day (no.)	8.08 (4.44)	15.20 (7.94)	0.001
Months of smoking (no.)	236.38 (155.91)	320.78 (109.03)	NS

Values indicate means (standard deviations) and the number of subjects. 1-Patients were defined as smokers if he/she was currently smoking and had been a smoker for at least eight years and as non-smokers if he/she had never smoked or quitted smoking longer than five years before the sampling.

The mean age of the study group was 48.37 ± 11.55 years; 62 individuals were male and 85 female. The perio group had significantly higher BPL, BOP, PPD and CAL values than the control group at both the full mouth and sampling site levels ($p<0.001$; Table 1). The number of smokers was significantly higher in the perio group than in the control group (41 and 13 patients, respectively, $p<0.001$; Table 1).

4.4.1. Comparison of GCF Cytokine Levels and Cytokine Ratio Values in Periodontally Healthy Individuals and Patients with Chronic Periodontitis

All the pro-inflammatory cytokines analysed (GMCSF, IL1alpha, IL1beta, IL6, IL12p40, IL17A, IL17F and TNFalpha), as well as four cytokines with anti-inflammatory effects (IFNgamma, IL2, IL3 and IL4), had significantly higher levels in the perio group than in the control group (adjusted *p*-value $\leq 1 \times 10^{-3}$; Table 2A).

Table 2A. Concentrations of cytokines in GCF that showed significant differences (adjusted *p*-values $\leq 1 \times 10^{-3}$ and $< 1 \times 10^{-5}$, respectively) between the control and perio groups.

Cytokine	Concentration in GCF (pg/ml) Median (IQR)		
	Control group	Perio group	Adjusted *P* value
GMCSF	150.24 (129.67)	247.24 (255.55)	7.29E-05
IL1alpha	30405.78 (20713.06)	148825.83 (221175.10)	2.64E-21
IL1beta	2881.75 (1958.43)	17947.50 (17308.08)	5.59E-21
IL6	166.71 (163.90)	313.45 (461.34)	3.77E-06
IL12p40	7.34 (5.65)	17.30 (11.06)	7.10E-12
IL17A	7.53 (9.38)	28.45 (25.83)	9.70E-19
IL17F	3.40 (7.01)	9.62 (15.47)	7.75E-09
TNFalpha	6.46 (13.49)	22.75 (15.95)	2.50E-04
IFNgamma	4.71 (3.85)	9.55 (9.60)	3.42E-07
IL2	9.96 (8.74)	14.96 (8.08)	0.001
IL3	54.26 (37.15)	99.60 (89.75)	1.23E-05
IL4	5.96 (21.42)	12.37 (47.02)	2.50E-04

Nineteen cytokine ratios showed significant differences between the control and perio groups (adjusted *p*-value <1 x 10^{-5}). Of these ratios, nine were based on the combination of two pro-inflammatory cytokines, which were: IL1alpha combined with GMCSF, IL12p40 and TNFalpha; and IL1beta combined with GMCSF, IL12p40, IL17F or TNFalpha, GMCSF/IL17A and IL17A/IL17F. The remaining ten ratios were based on the combination of one pro-inflammatory cytokine and one cytokine with anti-inflammatory effects. These were: IL1alpha/IL2; IL1alpha/IL3; IL1alpha/IFNgamma; ILbeta/IL2; ILbeta/IL3; ILbeta/IL4; ILbeta/IFNgamma; IL17A/IL2; IL17A/IL3; and IL17A/IFNgamma. All these cytokine ratios, except for GMCSF/IL17A, had significantly higher values in the perio group than in the control group (Table 2B).

Table 2B. Concentrations of cytokine ratios in GCF that showed significant differences (adjusted *p* values ≤1 x 10-3 and <1 x 10-5, respectively) between the control and perio groups.

Cytokine Ratio	Concentration in GCF (pg/ml) Median (IQR)		
	Control group	Perio group	Adjusted *P* value
GMCSF/IL17A	26.57 (25.02)	7.76 (14.82)	2.80E-08
IL1alpha/GMCSF	218.44 (198.78)	631.24 (1434.02)	1.03E-11
IL1alpha/IL12p40	4531.12 (3831.16)	6849.23 (17879.96)	2.78E-06
IL1alpha/TNFalpha	4524.95 (5930.96)	8427.24 (22799.21)	7.71E-06
IL1beta/GMCSF	19.52 (24.42)	67.75 (97.45)	1.18E-12
IL1beta/IL12p40	402.43 (516.02)	844.61 (1378.24)	2.83E-08
IL1beta/IL17F	662.10 (964.59)	1446.37 (3287.34)	1.62E-06
IL1beta/TNFalpha	468.80 (583.89)	854.64 (1669.85)	2.49E-07
IL17A/IL17F	0.88 (2.46)	2.31 (4.79)	1.88E-06
IL1alpha/IL2	3279.43 (3601.17)	8890.94 (17774.01)	1.93E-14
IL1alpha/IL3	630.99 (759.95)	2262.85 (5590.86)	1.42E-10
IL1alpha/IFNgamma	7728.27 (4744.27)	18426.38 (52652.97)	3.05E-12
IL1beta/IL2	260.08 (210.23)	1249.52 (1480.05)	4.16E-14
IL1beta/IL3	66.56 (87.48)	206.71 (469.16)	8.04E-11
IL1beta/IL4	536.43 (702.25)	1530.57 (2756.95)	7.25E-06
IL1beta/IFNgamma	805.90 (387.53)	2084.77 (5776.57)	5.54E-13
IL17A/IL2	0.64 (0.58)	1.96 (1.41)	5.54E-13
IL17A/IL3	0.13 (0.12)	0.34 (0.32)	4.80E-11
IL17A/IFNgamma	1.81 (1.03)	3.14 (2.03)	4.20E-12

4.4.2. Predictive Modelling of Chronic Periodontitis Based on GCF Cytokine Levels and Cytokine Ratios: Model Selection; Discrimination and Classification Measures; Determination of Diagnostic Thresholds; and Internal Validation

There were three cytokine-based models and three cytokine ratio-based models, which had an apparent AUC ≥0.85 for both non-smokers and smokers**.** These models were IL1alpha, IL1beta, IL17A, IL1alpha/IL2, IL1beta/IL2 and IL17A/IL2. Apparent and bc-percentages of discrimination and classification of the six predictive models are described in Tables 3A and 3B.

Table 3A. Apparent and bias-corrected measures of discrimination and classification of the models based on cytokines for both smokers and non-smokers.

Cytokine Model	Smoking Status	AUC	ACC (%)	SENS (%)	SPEC (%)	PPV (%)	NPV (%)
IL1alpha	Smoker	0.966 0.951	92.5 89.4	100.0 97.1	69.2 65.8	91.1 89.9	100.0 92.6
	Non-smoker	0.959 0.958	93.5 92.4	87.5 85.8	96.7 96.0	93.5 92.1	93.7 92.8
IL1beta	Smoker	0.968 0.945	94.4 90.7	97.5 96.6	84.6 71.1	95.2 91.9	92.3 88.8
	Non-smoker	0.944 0.943	94.6 94.1	90.6 89.5	96.7 96.5	93.7 93.4	95.2 94.6
IL17A	Smoker	0.940 0.912	92.5 90.3	95.1 93.9	84.6 79.1	95.2 93.6	85.7 82.1
	Non-smoker	0.914 0.914	88.1 86.8	78.1 76.1	93.4 92.5	86.2 84.3	89.2 88.2

In each cell, the first value refers to the apparent performance measures and the second to the corrected performance measures by the level of optimism, calculated using a bootstrap procedure.

The cytokine-based models had AUC and bc-AUC values ≥0.940 and ≥0.912, respectively, and the cytokine ratio-based model values were ≥0.857 and ≥0.834, respectively. The bc-ACC range derived from the cytokine-based models was 86.8%-94.1%, and that of the cytokine ratio-based models was 72.9%-88.7%, with IL17A and IL17A/IL2 being the biomarkers with the lowest bc-ACC values in both smokers

and non-smokers. The 95% CIs of the model coefficients and those of the performance measures are detailed in Appendices S3 and S4.

Table 3B. Apparent and bias-corrected measures of discrimination and classification of the models based on cytokine ratios for both smokers and non-smokers.

Cytokine Ratio Model	Smoking Status	AUC	ACC (%)	SENS (%)	SPEC (%)	PPV (%)	NPV (%)
IL1alpha/IL2	Smoker	0.878 0.868	85.1 81.1	100.0 99.0	38.4 29.5	83.6 80.2	100.0 99.0
	Non-smoker	0.911 0.905	88.1 85.1	84.3 80.0	90.1 88.6	81.8 78.6	91.6 89.2
IL1beta/IL2	Smoker	0.906 0.896	92.5 88.7	95.1 94.7	84.6 76.3	95.1 91.1	85.7 82.1
	Non-smoker	0.886 0.881	84.9 79.5	81.2 70.6	86.8 84.3	76.4 72.3	89.8 84.1
IL17A/IL2	Smoker	0.955 0.948	92.5 87.2	100.0 98.6	69.2 51.4	91.1 86.5	100.0 95.7
	Non-smoker	0.857 0.834	80.6 72.9	87.5 81.7	77.0 68.3	66.6 54.3	92.3 89.1

In each cell, the first value refers to the apparent performance measures and the second to the corrected performance measures by the level of optimism, calculated using a bootstrap procedure.

The periodontitis probability range for the median ACC values varied between 23% and 51%. The cytokine thresholds in pg/ml for the median ACC values (and those for the 90% CIs of the ACC values) for smokers and non-smokers were, respectively: IL1alpha model: 46099 (37495-64161) and 65644 (51310-76700); IL1beta model: 4732 (3705-6459) and 5827 (4721-7532); IL17A model: 11.03 (7.28-15.22) and 17.13 (13.10-22.53); IL1alpha/IL2 model: 4210 (3164-5648) and 7118 (4798-10166); IL1beta/IL2 model: 260 (63-487) and 628 (348-897); and IL17A/IL2 model: 0.810 (0.707-1.132) and 1.919 (1.073-3.489). The range of cytokine thresholds represented around 9-13% of the IL or ratio measurement range, except for IL17/IL2 for non-smokers (30%). Compared to the non-smokers, the smokers had lower diagnostic thresholds on all the predictive models for both apparent ACC values and ACC values obtained by bootstrapping (Figures 2-4).

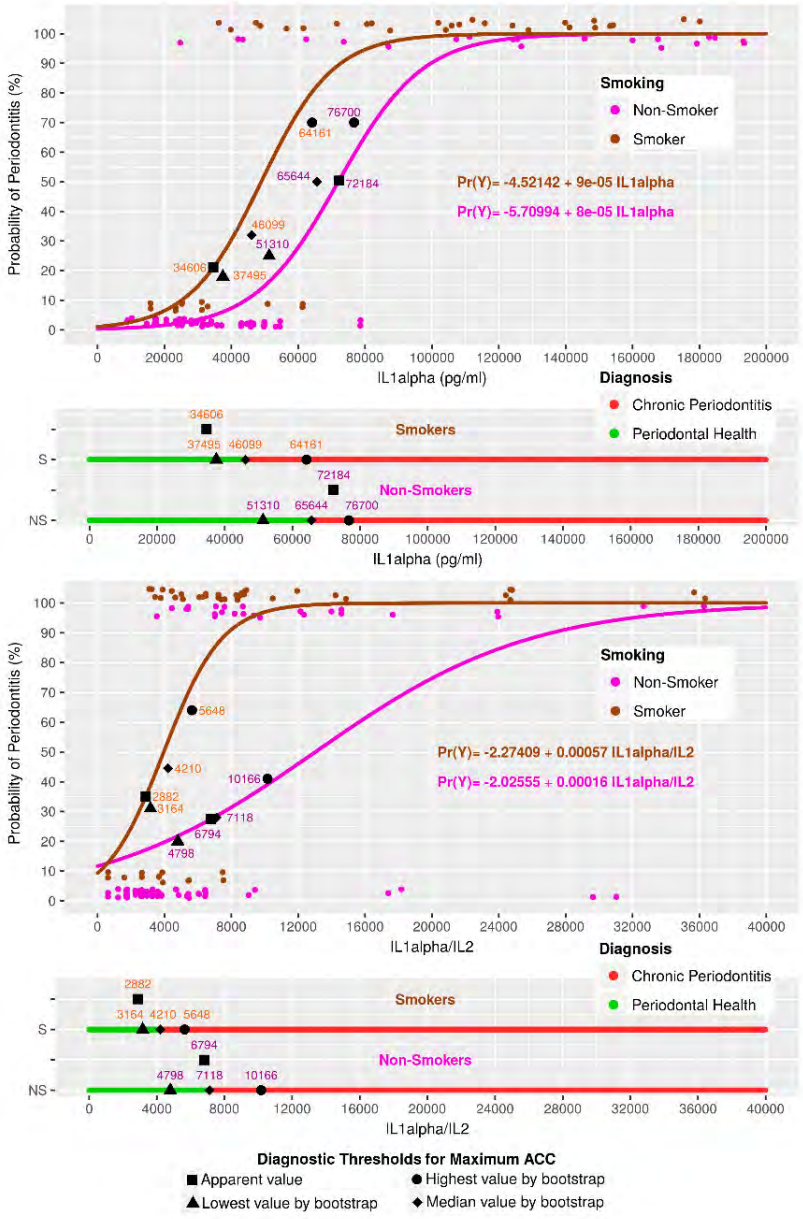

Figure 2. Model curves based on IL1alpha and IL1alpha/IL2, defining the diagnostic thresholds for apparent and median ACC values, as well as those thresholds for the 90% CIs of the ACC values.

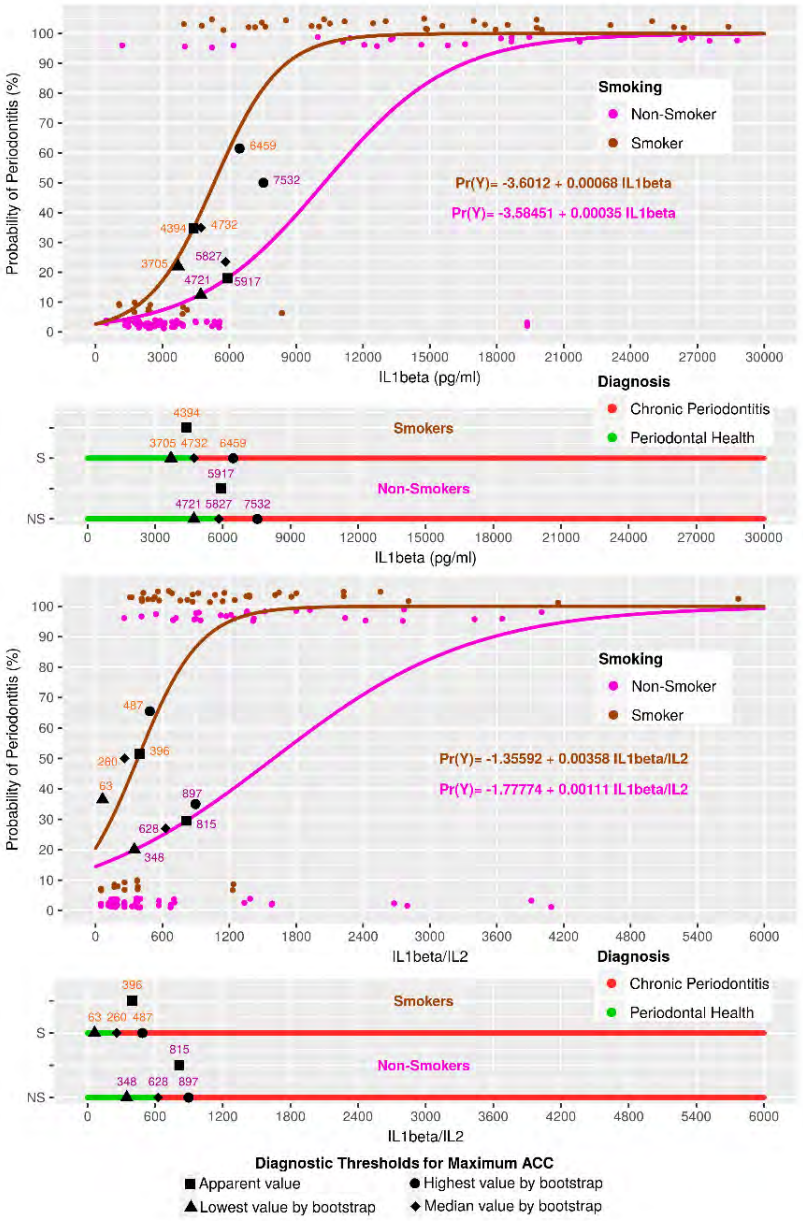

Figure 3. Model curves based on IL1beta and IL1beta/IL2, defining the diagnostic thresholds for apparent and median ACC values, as well as those thresholds for the 90% CIs of the ACC values.

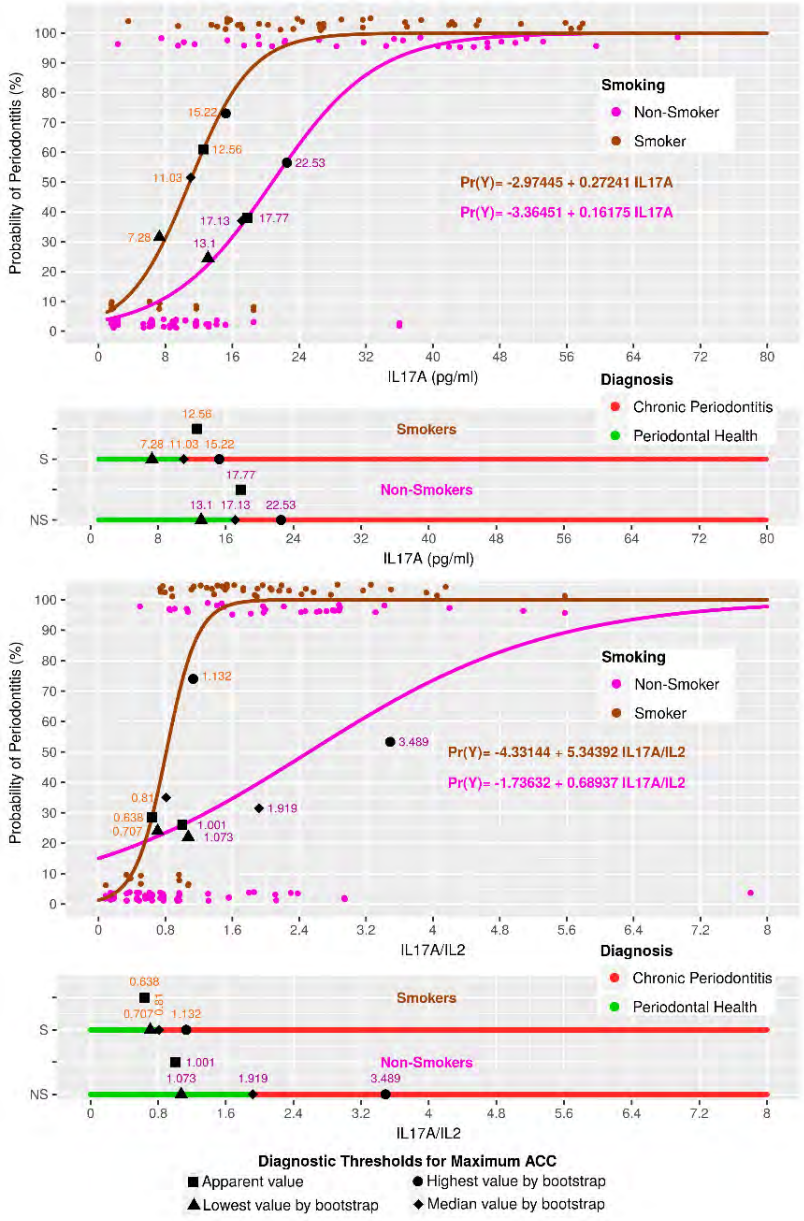

Figure 4. Model curves based on IL17A and IL17A/IL2, defining the diagnostic thresholds for apparent and median ACC values, as well as those thresholds for 90% CIs of the ACC values.

4.5. Discussion

4.5.1. High Cytokine Concentrations and Cytokine Ratios in the Gingival Crevicular Fluid of Patients with Chronic Periodontitis

As mentioned in the Introduction section, there has been a failure to study a broader spectrum of cytokines that may directly influence the local inflammatory response in different types of periodontitis [38]. The present series is the first comparative analysis of more than 50 cytokine ratios derived from the simultaneous quantification of 16 cytokines with different roles in the pathophysiology of chronic periodontitis [7,8]. It should be noted that an unusually strict corrected significance value was applied (adjusted p value $<1 \times 10^{-5}$) in order to select the cytokine ratios with the most significant impact on chronic periodontitis. This statistical decision conditioned the ratios considered to be non-significant and significant. As a consequence, comparisons with the contributions of other authors must be interpreted with caution.

Although very few authors have investigated the ratios between pro-inflammatory cytokines in periodontitis [39-41], up to eight pro-inflammatory cytokine ratios showed significant differences in periodontal patients. Although we detected significantly elevated levels of all the pro-inflammatory cytokines analysed, IL1alpha and IL1beta were the most important biomarkers in terms of increased concentration associated with the disease. This resulted in the ratios based on IL1alpha combined with GMCSF, or IL12p40 and IL1beta combined with GMCSF, IL12p40, IL17F or TNFalpha, showing significantly higher values in the periodontal patients. In agreement with the results reported by Awang et al. [42], we also obtained a significantly elevated IL17A/IL17F ratio in patients with chronic periodontitis. Interestingly, in this series, and unlike the other pro-inflammatory cytokine ratios, the GMCSF/IL17A ratio had significantly lower values in the periodontal patients, representing the first evidence of the impact of this ratio in the pathogenesis of periodontitis.

Most previous studies have focused on the analysis of ratios between pro-inflammatory and anti-inflammatory cytokines, or vice versa, in periodontal diseases, with IL1beta/IL10 and IL11/IL17 being the most evaluated [14,39,43-46]. In the present series, up to nine ratios based on the combination of one pro-inflammatory cytokine (IL1alpha, ILbeta or IL17A) and one cytokine with anti-inflammatory effects (IFNgamma, IL2, IL3 or IL4) showed significantly higher values in the periodontal patients. These results were due to the higher mean concentrations of pro-inflammatory cytokines compared to the levels presented by anti-inflammatory cytokines. In contrast to the findings of Stadler et al. [47], these mediators also showed a significant mean increase associated with chronic periodontitis. On the other hand, applying multivariate predictive modelling techniques, we have previously demonstrated that the extent of the periodontitis-associated imbalance between IL1alpha, ILbeta or IL17A (acting as enhancers) and IFNgamma, IL2, IL3 or IL4 (acting as protectors) was associated with a particular probability of having chronic periodontitis [25].

We have not found any articles that would enable us to compare our findings on ratios between IL1alpha and different anti-inflammatory cytokines. Regarding the ratios between ILbeta and other anti-inflammatory cytokines, some authors have observed that the ILbeta/IL10 ratio was increased in the GCF or gingival tissue of patients with aggressive periodontitis or chronic periodontitis [14,43,44] and that this ratio was significantly reduced after periodontal therapy [44]. However, after studying these papers in detail, those results can be attributed mainly to significantly higher mean levels of IL1beta, while the levels of IL10 showed non-significant individual variations. These results obtained *in vivo* call into question the importance of this ratio in the pathogenesis of periodontitis. Likewise, in the present study, no significant differences in IL10 levels between the controls and periodontal patients were detected, and therefore the IL1beta/IL10 ratio was not evaluated. However, it should be noted that IL10 acquired a more significant role as an anti-inflammatory cytokine within a two-biomarker predictive model, as this increased the capacity of IL1beta to discriminate the chronic periodontitis state [25].

In contrast, in the present series, we observed that other ratios, such as IL1beta/IFNgamma, ILbeta/IL2, ILbeta/IL3 and ILbeta/IL4, may play an essential role in quantitative terms in chronic periodontitis. Several studies have revealed that the IL11/IL17 ratio was reduced in patients with chronic and aggressive periodontitis [45,46,48], although other authors have described conflicting findings [39]. In the present study, other ratios such as IL17A/IFNgamma, IL17A/IL2, IL17A/IL3 and IL17A/IL4 had significantly higher values in the periodontal patients, reflecting their impact on chronic periodontitis.

Consequently, to the best of our knowledge, this study is the first time where evidence is provided on a high number of ratios between pro-inflammatory cytokines or pro-inflammatory and anti-inflammatory cytokines that, due to their performance in GCF samples, could be biomarkers associated with chronic periodontitis. Future research is required to clarify the relevance of these ratios in the chronic periodontitis pathogenesis.

4.5.2. High Predictive Ability of GCF Cytokine Levels and Cytokine Ratios for the Diagnosis of Chronic Periodontitis

Due to the complex characteristics of cytokine networks [49], whether cytokines in GCF may show an acceptable ability to discriminate chronic periodontitis from periodontal health is questioned. However, this affirmation is supported by little evidence, as there are very few studies that have evaluated the predictive properties of cytokines in chronic and aggressive periodontitis using an appropriate experimental design [19,20]. The current series reveals the first results on the predictive ability of cytokines and cytokine ratios for the diagnosis of chronic periodontitis, differentiating between smokers and non-smokers. Moreover, internal validation was carried out for the first time on the predictive parameters obtained, as recommended in the TRIPOD guidelines [24].

In this study, concerning individual cytokines, and corroborating observations published previously by our research group [25], there were three models consisting of IL1alpha, IL1beta and IL17A, which presented a bc-AUC>0.90 for both smokers and non-smokers. According to experts in the field [33], these high AUC values indicate that these pro-inflammatory cytokines have a great capacity to discriminate the disease condition. Consequently, these pro-inflammatory cytokines were associated with elevated bc-ACC percentages: 90.7% (for IL1beta), 90.3% (for IL17A) and 89.4% (for IL1alpha) in smokers; and 94.1%, 86.8% and 92.4%, respectively, in non-smokers. Findings on IL1's high predictive ability are consistent with those previously described by Baeza et al. [20], while IL17's findings represent the first evidence of a strong diagnostic capability. In our opinion, our results on the high predictive potential of these cytokines are comparable to those found for other well-known biomarkers, such as different metalloproteinases [20].

We evaluated the cytokine ratios using predictive modelling techniques to identify a set of biomarkers that guarantee high diagnostic predictability [16]. In this sense, we obtained three ratio-based models consisting of IL1alpha/IL2, IL1beta/IL2 and IL17A/IL2, which presented a bc-AUC>0.80 for both smokers and non-smokers. These bc-AUC values, although lower than those detected in individual cytokines, were also very high, revealing that these cytokine ratios were associated with an excellent ability to discriminate periodontitis patients [33].

For the first time in the literature, we have defined specific thresholds with diagnostic potential for each smoking status. These were derived from cytokine- and cytokine ratio-based predictive models and their validity was verified given that the apparent ACC and median ACC values obtained from the bootstrap approaches were similar. On the other hand, the range of thresholds obtained by bootstrapping represented only around 9-13% of the measurement range of the biomarker (except for the IL17A/IL2 ratio in non-smokers). Accordingly, the upper and lower thresholds of these ranges would ensure optimal diagnostic classification. In line with the trend of

attempting to discover biomarkers to improve the clinical diagnosis of periodontal diseases [16-18], the determination of these specific thresholds could represent a first step in the design and construction of chronic periodontitis diagnostic kits for use in clinical practice.

As smoking is a well-established traditional risk factor for chronic periodontitis [50,51], we demonstrated previously from a predictive perspective that smoking status increases the probability of having chronic periodontitis by 15-20% [25]. Interestingly, in the present series, smokers had lower diagnostic thresholds than non-smokers. At a biochemical level, this justifies what is observed at a clinical level, i.e. the presence of a less intense inflammatory reaction in smoking-associated periodontitis, indicating that smoking may have an immunosuppressant effect [50]. Secondly, it reveals the convenience of designing biomarker studies for predicting periodontal diseases differentiating by smoking status, especially if the diagnostic thresholds are to be defined.

If the models shown in Figures 2, 3 and 4 on the individual cytokines had similar discrimination or performance characteristics, other criteria for selecting the best molecules that will allow the construction of a possible diagnostic kit are as follows: 1) Relatively high range of concentrations in the healthy group; 2) Relatively high minimum measurement values (minimum sensitivity of the measurements); 3) The transition range between healthy and periodontal patients should be narrow, to avoid diagnostic uncertainty, but not too narrow to avoid false positives and false negatives.

The range of concentrations in the healthy group is high in IL1alpha (10000-50000 pg/ml), narrower in IL1beta (1000-5000 pg/ml), and very narrow in IL17A (4-16 pg/ml). In IL17A, a lower value is needed to detect the healthy ones than in the other molecules; that is, a higher sensitivity is needed to measure this molecule. The transition range is too narrow in IL17A. In IL1alpha it is excessively large, and we can say that the optimal transition would be in IL1beta. The transition zone is indicated between the triangle and the circle in each model. In IL1beta non-smokers, there is 7.960 pg/ml transition

range, in IL1alpha, there is 44.795 pg/ml, and in IL17A, there is 24.26 pg/ml.

It seems that the first candidate would be IL1beta, as it presents a narrower range of transition between healthy and periodontal patients since the remaining characteristics are similar to IL1alpha. The last model to be chosen for the construction of a possible diagnostic kit would be IL17A.

Our research has some limitations. Although we are in a scenario of small data, the sample size used allowed certain metrics of model performance were estimated with an acceptable precision [24]; also, a strict model selection criterion (apparent AUC value ≥0.85) was applied for both non-smokers and smokers. An internal validation process was carried out using bootstrap techniques, with the aim being to counteract the prediction that the study's accuracy is only measured in the samples that generated the model equations [24]. Although the results derived from the internal validation were quite optimal, the predictive parameters and diagnostic thresholds obtained from our models should be evaluated in an external cohort of patients (including using calibration analyses) to verify whether our findings are applicable universally. Likewise, it would be interesting to investigate the diagnostic accuracy of the cytokine ratios described in the present study on salivary samples.

In conclusion, a high number of previously undescribed GCF cytokine ratios are elevated in patients with chronic periodontitis, evidencing disease-associated imbalances between cytokines with pro-inflammatory and anti-inflammatory effects. IL1alpha, IL1beta and IL17A, and their ratios with IL2 are excellent diagnostic biomarkers in GCF for distinguishing systemically healthy subjects with chronic periodontitis patients from periodontally healthy individuals, independently of smoking status. Cytokine thresholds in GCF with diagnostic potential are defined, showing that smokers have lower threshold values than non-smokers.

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OBJECTIVE 5

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Objective 5. Diagnostic Accuracy of IL1beta in Saliva: The Development of Predictive Models for Estimating the Probability of the Occurrence of Periodontitis in Non-Smokers and Smokers

5.1. Abstract

Aim: To obtain salivary interleukin (IL) 1beta-based models to predict the probability of the occurrence of periodontitis, differentiating by smoking habits.

Materials and Methods: A total of 141 participants were recruited, including 62 periodontally healthy controls, as well as 79 subjects affected by periodontitis. Fifty of those in this latter group were given non-surgical periodontal treatment and showed significant clinical improvement at two months. IL1beta was measured in the salivary samples using the Luminex 200™ instrument. Predictive models were obtained using binary logistic regression to differentiate untreated periodontitis from periodontal health (first modelling) and untreated periodontitis from treated periodontitis (second modelling), distinguishing between non-smokers and smokers. The area under the curve (AUC) and numerous classification measures were obtained.

Results: In the first modelling, IL1beta presented AUC values of 0.830 for non-smokers and 0.689 for smokers. In the second, the predictive models of non-smokers and smokers revealed AUC values of 0.671 and 0.708, respectively.

Conclusions: Salivary IL1beta has an excellent diagnostic capability when it comes to distinguishing systemically healthy patients with untreated periodontitis from those who are periodontally healthy,

although this discriminatory potential is reduced in smokers. The diagnostic capacity of salivary IL1beta remains acceptable for differentiating between untreated and treated periodontitis.

Clinical relevance: Saliva is the mirror of the body and, as it can be collected easily and non-invasively, could be useful for screening and monitoring tests for periodontitis based on quantifiable biomarkers. IL1beta has an excellent diagnostic capability when it comes to differentiating between patients with untreated periodontitis and those who are periodontally healthy, although this discriminatory potential is reduced in smokers. Its capacity to distinguish between untreated and treated periodontitis is also acceptable. The diagnostic threshold values of salivary IL1beta in smokers are lower than in non-smokers in different clinical settings, evidencing the importance of determining the specific diagnostic thresholds of this biomarker in both smoking conditions. The salivary level of IL1beta is a valuable tool for diagnosing periodontitis in systemically healthy subjects.

5.1.1. Keywords

Interleukin 1beta; saliva; periodontitis; diagnostic accuracy; sensitivity; specificity; predictive values; prevalence

5.1.2. Declaration of Conflict of Interest

The doctoral candidate and the rest of the authors of the present study declare that they have no conflict of interest concerning the objectives proposed in this paper.

5.1.3. Funding

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5.2. INTRODUCTION

Periodontitis is considered to be the sixth most prevalent disease worldwide, with more than 740 million people estimated to be affected by this condition [1]. The early diagnosis of periodontitis is a fundamental element for the success of the therapy, as the progression of the disease causes an irreversible loss of periodontal structures [2]. A periodontitis patient remains so forever, even following successful treatment, and requires life-long supportive care to prevent any recurrence of the disease [3].

Traditional clinical parameters are the best measures currently available for diagnosing and monitoring periodontitis [4]. However, these do not provide reliable information on the current activity of the disease and its future progression. Moreover, they are error-prone, time-consuming and often poorly tolerated by patients [5,6].

It is rightly said that saliva is the mirror of the body and, as it can be collected in an easy and non-invasive way, could be valuable for screening and monitoring periodontitis [6]. Many researchers are currently focusing their efforts on identifying objectively quantifiable biomarkers in saliva that can reliably reflect the subgingival pathophysiological status associated with periodontitis [7-9]. Cytokines play a crucial role in initiating and sustaining the inflammatory immune response by stimulating the production of secondary mediators that are responsible for the degradation of connective tissue and osteoclastic bone resorption [10]. Specifically, interleukin (IL) 1beta is a pro-inflammatory cytokine that plays a significant role in the pathogenesis of periodontitis [11].

In order to assess the diagnostic capacity of a biomarker, it is necessary to design a diagnostic precision study that reveals discrimination and classification measures of a clinical condition [12]. IL1beta was the first cytokine to be quantified in the gingival tissue of patients with chronic periodontitis [13], and in objective 3, we demonstrated that IL1beta is an outstanding biomarker for distinguishing systemically healthy patients with chronic periodontitis

from periodontally healthy individuals. However, as reflected in Objective 2, very few studies have been published on the diagnostic accuracy of IL1beta in saliva, and none of them has evaluated the impact of a patient's "smoking habit" on this diagnostic capability [14-20].

Consequently, the objectives of this cross-sectional study were to:

1) Compare the levels of IL1beta detected in saliva in systemically healthy individuals with health periodontal to those of patients with periodontitis, and in periodontitis patients before and after they receive conventional treatment.

2) Obtain predictive models based on salivary IL1beta that could be used to differentiate between treated and untreated periodontitis patients and periodontally healthy individuals, distinguishing them by smoking status and developing corresponding clinical application nomograms for the specification of different diagnostic thresholds.

3) Describe the classification measures derived from salivary IL1beta-based models and evaluate the clinical consequence of this cytokine for the diagnosis of untreated periodontitis according to the different prevalences of the disease.

5.3. MATERIALS AND METHODS

5.3.1. Selection of Study Groups

A convenience sample of 141 eligible participants, comprising 62 periodontally healthy controls (control group) and 79 subjects affected by untreated periodontitis (untreated perio group), were recruited from 320 consecutive patients in the general population who were referred to the School of Medicine and Dentistry (Universidade de Santiago de Compostela, Spain) for an assessment of their oral health status from 2016-2018. Patients were selected if they fulfilled the following inclusion criteria: 1) age 30 to 75; 2) the presence of at least 18 natural teeth; 3) no previous periodontal treatment; 4) no medical history of

diabetes mellitus, hepatic or renal disease, or other severe medical conditions or transmittable diseases; 5) no intake of systemic antimicrobials during the previous six months; 6) no intake of anti-inflammatory medication in the previous four months; 7) no routine use of oral antiseptics; 8) no history of alcohol or drug abuse; 9) no pregnancy or breastfeeding; 10) no presence of implants or orthodontic appliances; 11) smoked for at least one year; 12) never smoked or had stopped more than three years ago.

Two experienced dentists performed all the periodontal diagnoses. The bleeding on probing (BOP) and the bacterial plaque level (BPL) were recorded for the full mouth on a binary scale (presence/absence) on six sites per tooth. The probing pocket depth (PPD) and clinical attachment level (CAL) were recorded throughout the mouth, also on six sites per tooth, using a PCP-UNC 15 probe. Standardised radiographs of all the teeth were obtained to assess the alveolar bone status.

The diagnosis of periodontitis was based on the clinical and radiographic information obtained. The control group included periodontally healthy patients who had: <25% BOP, no location with PPD ≥4 mm, and no radiographic evidence of alveolar bone loss. The untreated perio group included patients who were diagnosed with periodontitis (stages II-IV) by applying the new, recently published, classification criteria [4,21]. The "smoking habit" was also evaluated using a questionnaire, with information collected on the extent of the habit (non-smoker, former smoker, current smoker, time spent as a former or current smoker, and the number of cigarettes consumed per day).

Of the 79 patients in the untreated perio group, 60 received conventional non-surgical treatment (scaling and root planning) and were clinically evaluated after two months (treated perio group). In the 2-month reevaluation, there were ten patients who showed no clinical improvement in terms of BOP and PPD and were excluded from the present study.

The study was conducted in accordance with the principles of the Declaration of Helsinki (revised in 2000) on studies involving human experimentation [22]. The study protocol was approved by the Galician Clinical Research Ethics Committee (registration number 2015/006; Appendix S1). TRIPOD and STARD guidelines on studies on diagnostic accuracy were applied; the TRIPOD checklist is displayed in Appendix S2 [23].

5.3.2. Collection of Salivary Samples and Quantification of Cytokines Using Multiplexed Bead Immunoassays

Unstimulated saliva samples were taken from each patient using the spitting method in the first ten days after intraoral exploration and two months after they received conventional periodontal treatment [24]. The subjects avoided practising any oral hygiene measure, eating, drinking or chewing gum from one hour before the collection of the saliva sample. The samples were stored at -80ºC until further biochemical analysis.

A single investigator blinded to the clinical data performed the quantitative cytokine analyses. Salivary levels of IL1beta were determined using a Milliplex® ultrasensitivity kit (Merck Chemicals and Life Science, S.A., Madrid, Spain) and the Luminex 200™ instrument (Luminex Corporation, Austin, Texas, USA). The IL1beta concentrations of the unknown samples (antigens in the salivary samples) were estimated from the standard curve using a 5PL algorithm and the xPONENT 3.1 software (Luminex Software, Inc.), and were expressed as pg/ml, adjusting for the dilution factor. The concentration ranges for the IL1beta were 0.49-2000 pg/ml. Samples below the detection limit (DL) of the assay were recorded as DL/2 [25].

5.3.3. Statistical Analysis

The statistical analyses were performed using the R software (version 3.4.3) [26]. In the calculation of the sample size, estimating a pre-determined AUC value of 0.850 with a given marginal error of 0.1 and 95% confidence level, the required sample size was 38 subject in each clinical condition, both non-smokers and smokers [27].

5.3.3.1. Comparison of the Clinical Characteristics and Salivary Levels of IL1beta between the Control and Periodontitis Subjects, and between the Latter Before and After Non-Surgical Periodontal Treatment

After applying the Shapiro-Wilks test and verifying the non-normal distribution of almost all the clinical variables, the Mann-Whitney U test was used to compare the quantitative clinical variables and the IL1beta levels between the control and untreated perio groups. The Fisher's exact test was used to assess the association of the qualitative variables between the two study groups. After non-surgical periodontal treatment, the paired Wilconxon test was applied to compare periodontal parameters and IL1beta levels between the untreated and treated perio groups. A significance level of $p<0.05$ was established.

5.3.3.2. Diagnostic Capability of Salivary Levels of IL1beta for the Detection of Untreated Periodontitis in Non-Smokers and Smokers: Obtaining Predictive Models and the Development of Nomograms

Figure 1 details the protocol used in the present study for the predictive modelling of salivary IL1beta for the diagnosis of untreated periodontitis. Predictive models were constructed by selecting IL1beta as a predictor variable, which was treated in its original scale. Two modelling phases were performed: in the first one, periodontally healthy patients were the "control condition", while in the second, the "control condition" was represented by the treated periodontal patients who had seen significant clinical improvement in terms of BOP and PPD. In both modelling phases, the "target condition" comprised patients with untreated periodontitis. These different models were

developed for both non-smokers and smokers (first modelling, n=76 and 65, respectively; second modelling -paired design-, n=60 and 40, respectively).

The capacity of the models to discriminate the presence of untreated periodontitis was evaluated through the area under the curve (AUC) value. It is worth noting that models with an AUC value equal to or higher than 0.70 are considered to be acceptable predictive models [28]. The AUC values and their corresponding 95% confidence intervals (CIs) obtained by bootstrapping were calculated using the pROC package, version 1.15.0 [29].

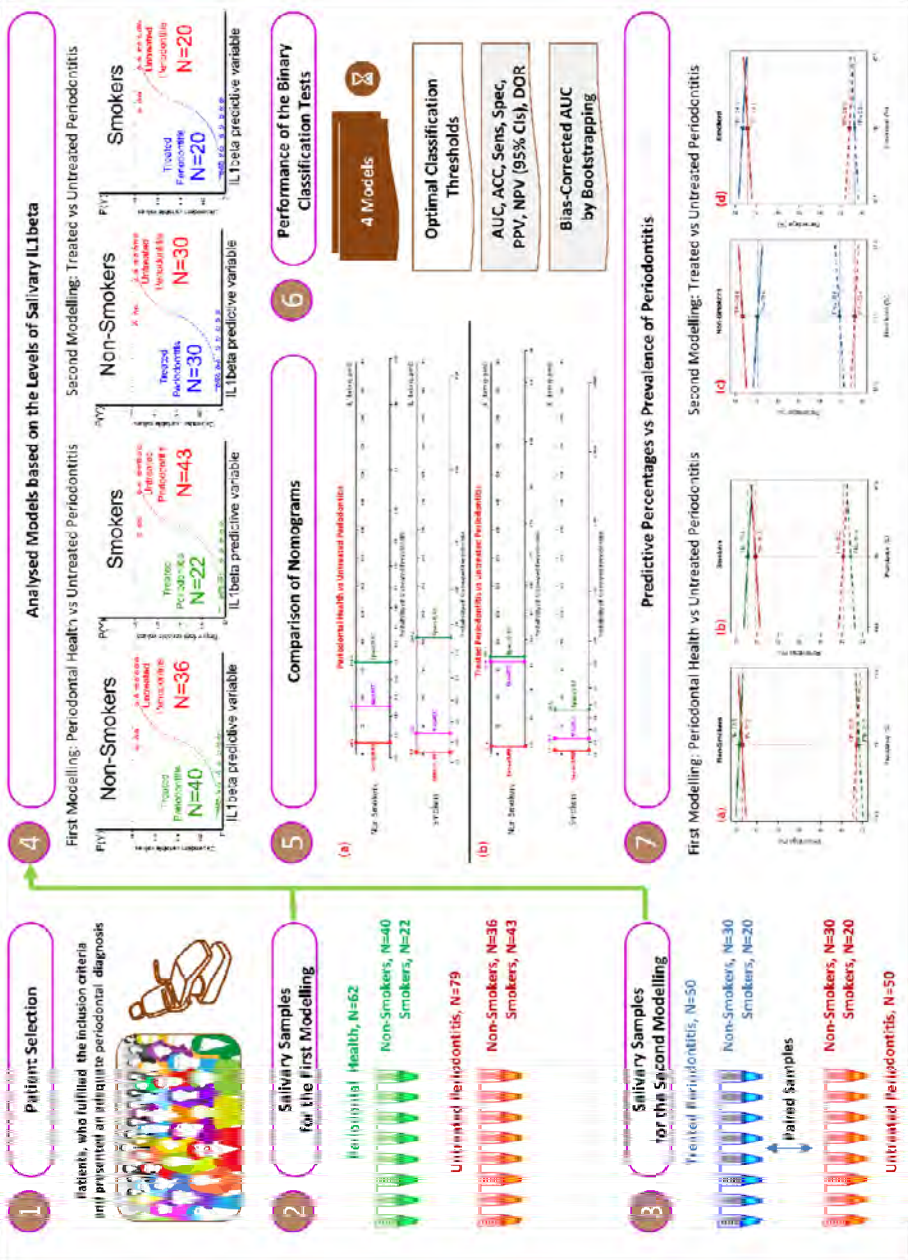

Figure 1. The protocol used for the predictive modelling of salivary IL1beta for the diagnosis of untreated periodontitis.

In order to validate the IL1beta-based predictive models internally, the mean optimism values concerning the discrimination measures were determined using "bootstrap" methods. Optimism is a measure that indicates the absolute magnitude of error associated with a model. It is obtained from the difference between the AUC values derived from models obtained with replacement sampling (1000 models) and their corresponding AUC values retrieved from the original model [30,31]. The bias-corrected AUC value (bc-AUC) was obtained from the corresponding apparent measure derived from the original sample minus its respective mean optimism value [30,31].

As a calibration measure for cytokine-based models, the Hosmer-Lemeshow test was applied using the ResourceSelection package, version 0.3-4 [32]. Significant values of $p<0.05$ are indicative of poorly calibrated models [33].

The nomograms were constructed graphically based on the results of the predictive analysis using the rms package, version 5.1-3.1 [34]. Being a nomogram derived from univariate models, the value of the predictor variable (IL1beta) corresponds to the probability that a patient has untreated periodontitis [35].

5.3.3.3. Determination of Diagnostic Thresholds of IL1beta in Non-Smokers and Smokers and Classification Measures

The optimal classification threshold for each model was defined as the one that provided the highest number of correct predictions (maximum accuracy -ACC-) and was determined using the PresenceAbsence package, version 1.1.9 [36]. Diagnostic thresholds for sensitivity and specificity values >90% were also calculated using the OptimalCutpoints package, version 1.1-4 [37].

Establishing the threshold for maximum ACC, several classification measures and their corresponding 95% CIs (obtained by bootstrapping) were calculated using the pROC package, version 1.15.0 [29]. These classification measures were: the ACC, the sensitivity (SENS), the specificity (SPEC), the positive predictive value (PPV) and the negative predictive value (NPV). The diagnostic odds ratio (DOR)

was calculated as a single diagnostic classification indicator, defined as the quotient of the probabilities of positivity in patients with the target condition in relation to the positivity probabilities in those with the control condition [38]. The guidelines established by de Luca Canto et al. [39] were used to interpret the classification parameters.

5.3.3.4. Clinical Consequences of IL1beta in Saliva for the Diagnosis of Untreated Periodontitis in Non-Smokers and Smokers According to the Prevalence of the Disease

Applying the recommendations previously established by experts in the field [40], we evaluated the clinical consequences of salivary IL1beta for diagnosing untreated periodontitis in non-smokers and smokers. The sensitivity and specificity data obtained from the different predictive models were extrapolated to a hypothetical cohort of 1000 patients, and the predictive values associated with the IL1beta and IL10 salivary tests were calculated for the different prevalences of periodontitis.

5.4. Results

5.4.1. Comparison of the Clinical Characteristics and Salivary Levels of IL1beta in the Control Subjects and the Periodontitis Patients, and in These Patients Before and After Non-Surgical Periodontal Treatment

Concerning the clinical parameters associated with the periodontal health status, the patients in the untreated perio group had significantly higher BPL, BOP, PPD and CAL values than those in the control group (p<0.001; Tables 1A and 1B). BOP and PPD parameters were significantly reduced in the periodontal patients after treatment compared to the pre-treatment periodontal condition, in both smokers and non-smokers (p<0.001; Tables 1A and 1B; Appendix S3).

Table 1A. Age, gender, smoking habit and clinical characteristics associated with periodontal status in the control and non-treated perio groups.

CLINICAL PARAMETERS	STUDY GROUPS		
	Control group (n=62)	**Non-treated Perio group (n=79)**	***P* Value**
Age (years)	39.47 (8.82)	48.94 (8.73)	<0.001
Gender			
Male	32	38	NS
Female	30	41	
Smoking habit			
Non-smokers	40	36	0.028
Smokers	22	43	
Cigarettes/day (no.)	8.68 (6.11)	16.92 (8.45)	0.008
Months of smoking (no.)	215.00 (135.23)	343.14 (120.49)	0.001
No. of teeth	28.05 (1.84)	23.76 (5.41)	<0.001
Full mouth			
BPL (%)	18.36 (16.25)	51.94 (28.85)	<0.001
BOP (%)	7.52 (8.67)	46.44 (20.87)	<0.001
PPD (mm)	2.08 (0.38)	3.61 (0.59)	<0.001
CAL (mm)	1.15 (0.40)	3.60 (1.25)	<0.001

Values indicate means (standard deviations) and number of subjects.

Table 1B. Age, gender, smoking habit and clinical characteristics associated with periodontal status in the perio group patients undergoing non-surgical periodontal treatment before and after treatment.

CLINICAL PARAMETERS	STUDY GROUPS		
	Treated Perio group (n=50)	**Non-treated Perio group (n=50)**	***P* Value**
Age (years)	50.24 (8.53)		NA
Gender			
Male	44		NA
Female	56		
Smoking habit			
Non-smokers	30		NA
Smokers	20		
Cigarettes/day (no.)	10.16 (11.02)		NA
Months of smoking (no.)	178.14 (175.65)		NA
No. of teeth	22.36 (4.65)		NA
Full mouth			
BPL (%)	35.28 (19.99)	45.88 (28.67)	<0.001
BOP (%)	28.24 (13.79)	53.58 (20.45)	<0.001
PPD (mm)	2.87 (0.52)	3.52 (0.67)	<0.001
CAL (mm)	3.16 (1.29)	3.39 (1.34)	0.043

Values indicate means (standard deviations) and the number of subjects.

In the patients in the untreated perio group, the salivary levels of IL1beta were significantly higher than those observed in the control group in both non-smokers and smokers (IL1beta medians: 202.59 and 101.44 pg/ml *versus* 16.49 and 23.65 pg/ml, respectively; $p \leq 0.01$ (Figures 2A and 2B).

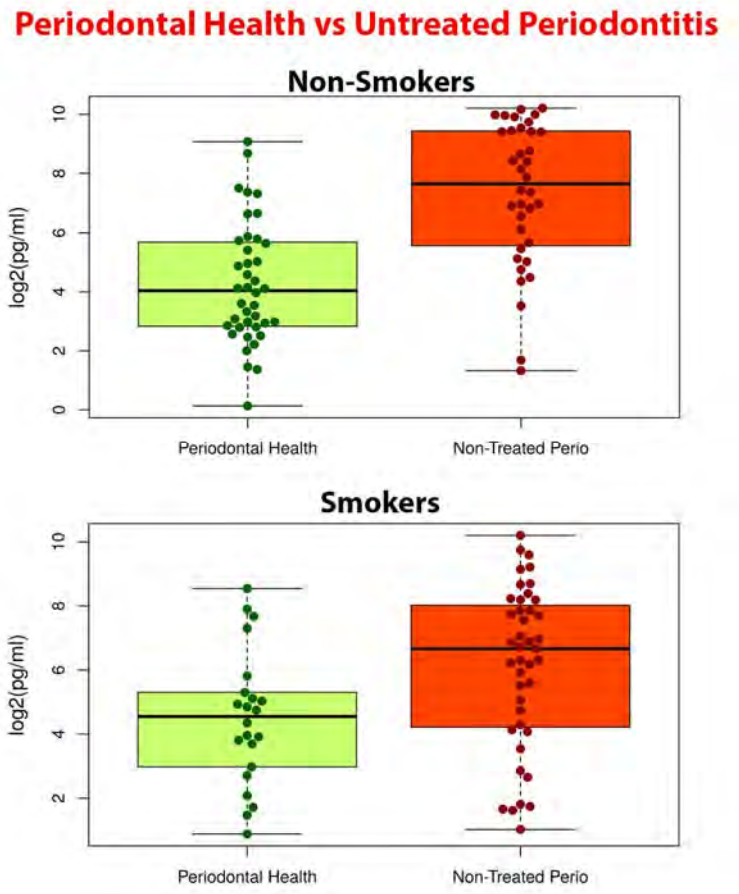

Figure 2A. Box plots of the levels of IL1beta salival (log2 pg/ml) between the periodontal health and untreated periodontitis groups, differentiating between non-smokers and smokers.

The salivary levels of IL1beta in the treated periodontal patients were significantly lower than those in these same patients before periodontal treatment, again in both non-smokers and smokers (respectively, IL1beta median: 44.34 and 16.74 pg/ml; and IL1beta median: 150.35 and 42.50 pg/ml: $p<0.001$ (Figures 2A and 2B).

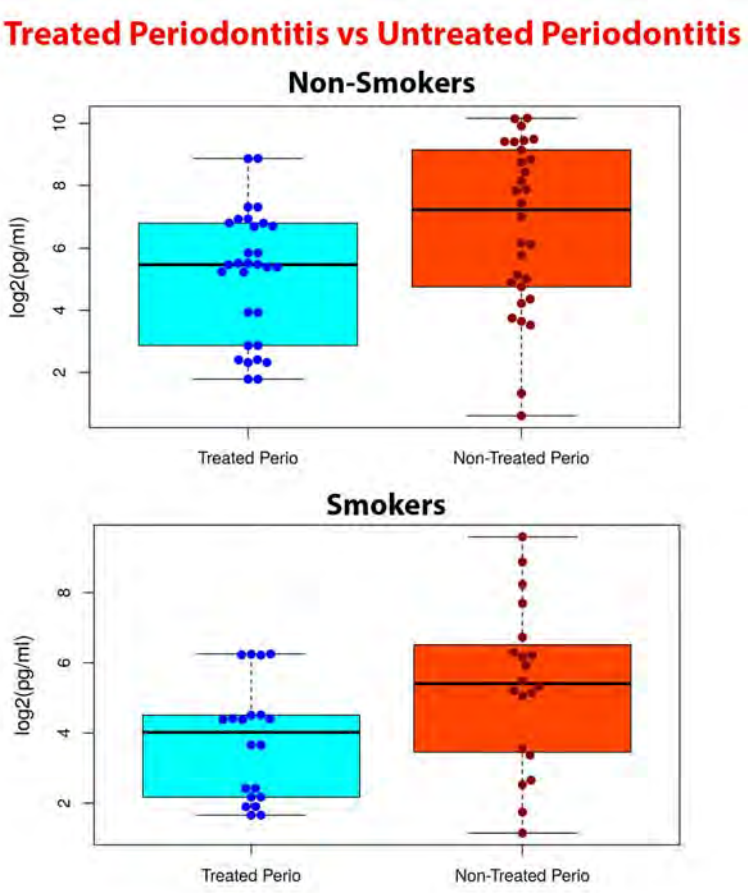

Figure 2B. Box plots of the levels of IL1beta salival (log2 pg/ml) between the treated and untreated periodontitis groups, differentiating between non-smokers and smokers.

5.4.2. Diagnostic Accuracy of Salivary Levels of IL1beta for the Detection of Untreated Periodontitis in Non-Smokers and Smokers: Obtaining Predictive Models and the Development of Nomograms

The IL1beta-based predictive model associated with the non-smoking condition revealed higher AUC values (0.830 *versus* 0.689) in the first phase of modelling; in the second phase, the predictive models presented AUC values in non-smokers and smokers of 0.671 and 0.708, respectively (Table 2; additional information on these models is detailed in Appendix S4). The Hosmer-Lemeshow test values were not significant in any of the predictive models (p>0.05), confirming their adequate calibration.

Table 2. Description of the salivary IL1beta-based models, including apparent and bias-corrected AUC values.

IL1beta-based model (untreated periodontitis/periodontal health)		**AUC**	**bc-AUC**
Non-Smoker	1.053 + 0.006 **IL1beta**	0.830	0.826
Smoker	0.0817 + 0.005 **IL1beta**	0.689	0.688
IL1beta-based model (untreated periodontitis/treated periodontitis)		**AUC**	**bc-AUC**
Non-Smoker	-0.690 + 0.004 **IL1beta**	0.671	0.666
Smoker	-0.794 + 0.018 **IL1beta**	0.708	0.701

In relation to the nomograms, in general, the higher a patient's salivary levels of IL1beta, the greater the probability that he/she suffered from untreated periodontitis. A comparison of the two nomograms derived from the first phase of modelling revealed that, for the same probability of suffering from untreated periodontitis, for example, for a 70% probability, the salivary levels of IL1beta that predict this probability are much higher in non-smokers than in smokers (300 pg/ml and 140 pg/ml, respectively); this finding was also present in the nomograms derived from the second modelling phase (for a 70% probability, the IL1beta values were 350 pg/ml for non-smokers and 90 pg/ml for smokers; Figures 3A and 3B).

5.4.3. Determination of Diagnostic Thresholds of Salivary IL1beta in Non-Smokers and Smokers and Classification Measures

The diagnostic thresholds for maximum ACC for salivary IL1beta were 84.76 pg/ml in non-smokers and 42.78 pg/ml in smokers; after non-surgical periodontal treatment, these thresholds for distinguishing untreated from treated periodontitis were 163.50 pg/ml and 27.66 pg/ml, respectively (Figures 3A and 3B). Diagnostic thresholds for sensitivity and specificity values ≥90% are also described in Figures 3A and 3B.

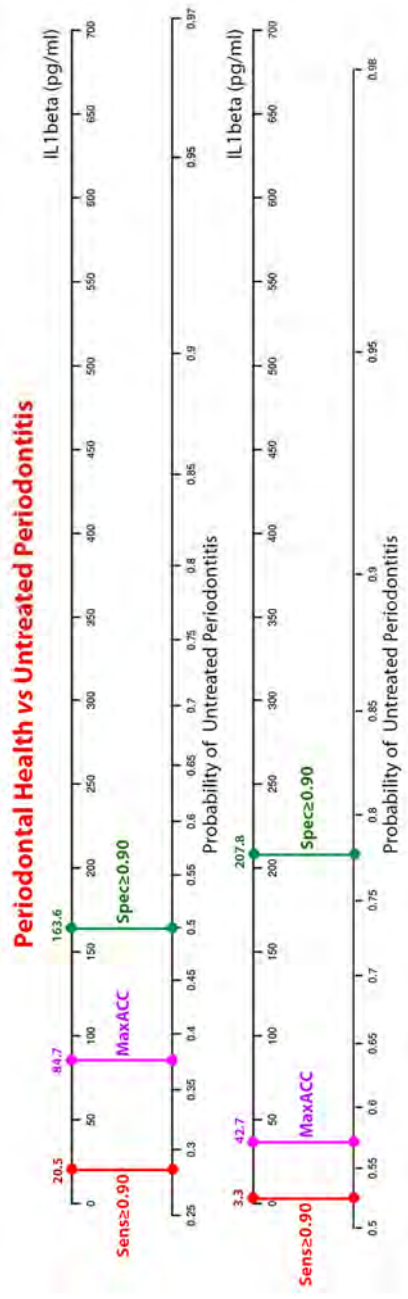

Figure 3A. Clinical application nomograms derived from the first phase of modelling (periodontal health *versus* untreated periodontitis), differentiating between non-smokers and smokers (upper and lower, respectively).

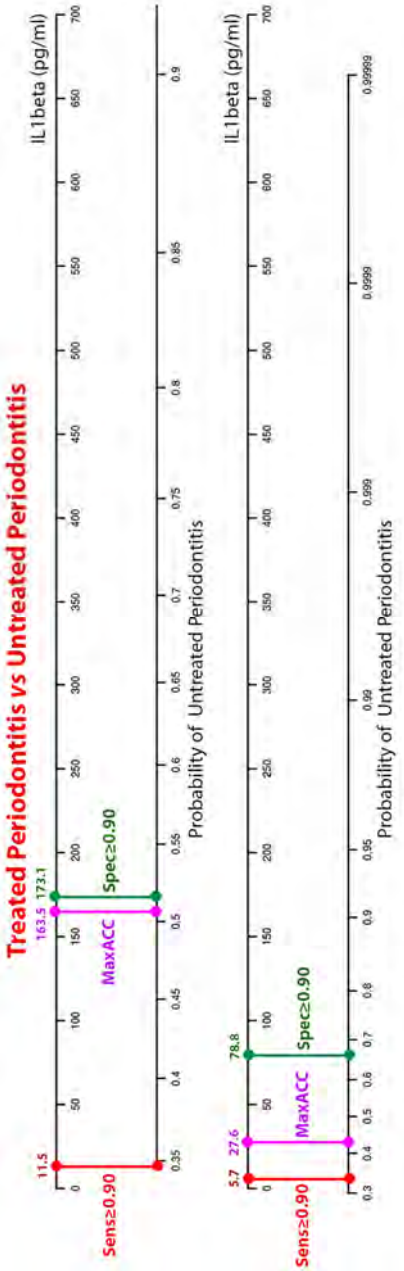

Figure 3B. Clinical application nomograms derived from the first phase of modelling (periodontal health *versus* untreated periodontitis), differentiating between non-smokers and smokers (upper and lower, respectively).

In the first modelling phase, the non-smokers had higher values for all the classification measures than the smokers, except for the PPV percentage. These values for both conditions ("no smoking" and "smoking") were, respectively: ACC, 77.63% and 70.76%; sensitivity, 72.22% and 67.44%; specificity, 82.50% and 77.27%; and DOR values, 12.25 and 7.04.

In the second modelling phase, the non-smokers had lower values of classification parameters than the smokers, especially in the sensitivity. These values for both conditions ("no smoking" and "smoking") were, respectively: ACC, 70% and 75%; sensitivity, 53.3% and 70%; specificity, 86.6% and 80%; and DOR values, 7.42 and 9.33 (Table 3). The 95% CIs of the different performance measures are detailed in Appendix S5.

Table 3. Measures of classification of the salivary derived from the salivary IL1beta-based models.

IL1beta-based Model (Untreated Periodontitis/Periodontal Health)						
Smoking status	**ACC (%)**	**SENS (%)**	**SPEC (%)**	**PPV (%)**	**NPV (%)**	**DOR**
Non-Smoker	77.6	72.2	82.5	78.7	76.7	12.25
Smoker	70.7	67.4	77.2	85.2	54.8	7.04
IL1beta-based Model (Untreated Periodontitis/Treated Periodontitis)						
Smoking status	**ACC (%)**	**SENS (%)**	**SPEC (%)**	**PPV (%)**	**NPV (%)**	**DOR**
Non-Smoker	70.0	53.3	86.6	80.0	65.0	7.42
Smoker	75.0	70.0	80.0	78.5	73.0	9.33

5.4.4 Clinical Consequence of Salivary IL1beta for Diagnosing Untreated Periodontitis in Non-Smokers and Smokers According to the Prevalence of the Disease

In relation to the clinical effectiveness of salivary IL1beta for diagnosing untreated periodontitis, and considering the sensitivity and

specificity values and a 45% prevalence of the disease [41,42], values of 77.2% and 70.8% for the total IL1beta positive tests in non-smokers and smokers, respectively, would indicate a true positive (untreated periodontitis patients); meanwhile, of the total IL1beta negative tests, a value of 78.4% in non-smokers and 74.4% in smokers would suggest a true negative (periodontally healthy patient) (Figures 4A and 4B).

In the second modelling phase (untreated /treated periodontitis), these values in non-smokers and smokers would be 76.6% and 74.1%, and 69.4% and 76.5%, respectively (Figures 4C and 4D).

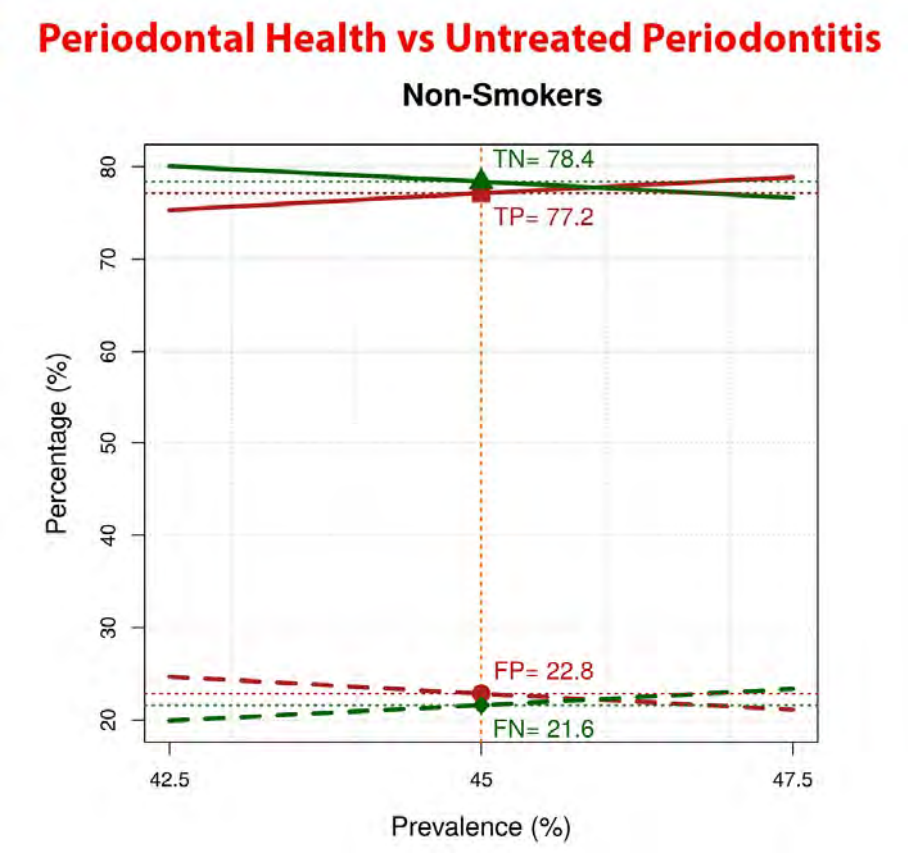

Figure 4A. Predictive percentages of salivary IL1beta biomarker for different prevalence values of periodontitis in non-smokers (first modelling).

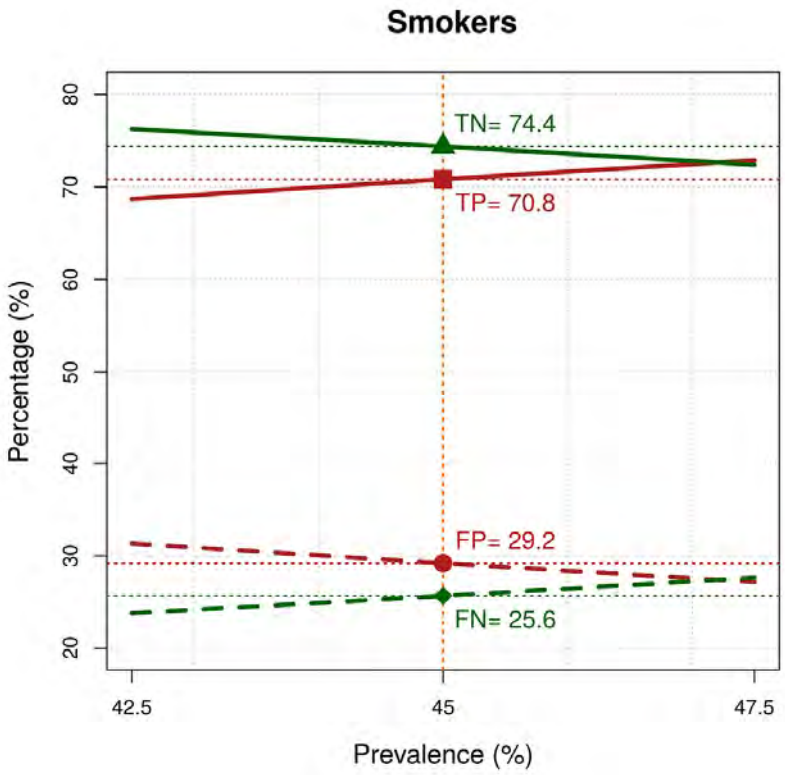

Figure 4B. Predictive percentages of salivary IL1beta biomarker for with different prevalence values of periodontitis in smokers (first modelling).

The continuous line indicates the true cases for the different prevalence of periodontitis (the green line, the true negatives; the red line, the true positives). The discontinuous line indicates false cases for the different prevalence of periodontitis (the green line, false negatives; the red line, false positives). TP: true positive, test is positive (indicates periodontitis and patient has periodontitis); FP: false positive, test is positive (indicates periodontitis but patient does not have periodontitis); TN: true negative, test is negative (indicates periodontitis not present and patient does not have periodontitis); FN: false negative, test is negative (indicates periodontitis not present but patient has periodontitis).

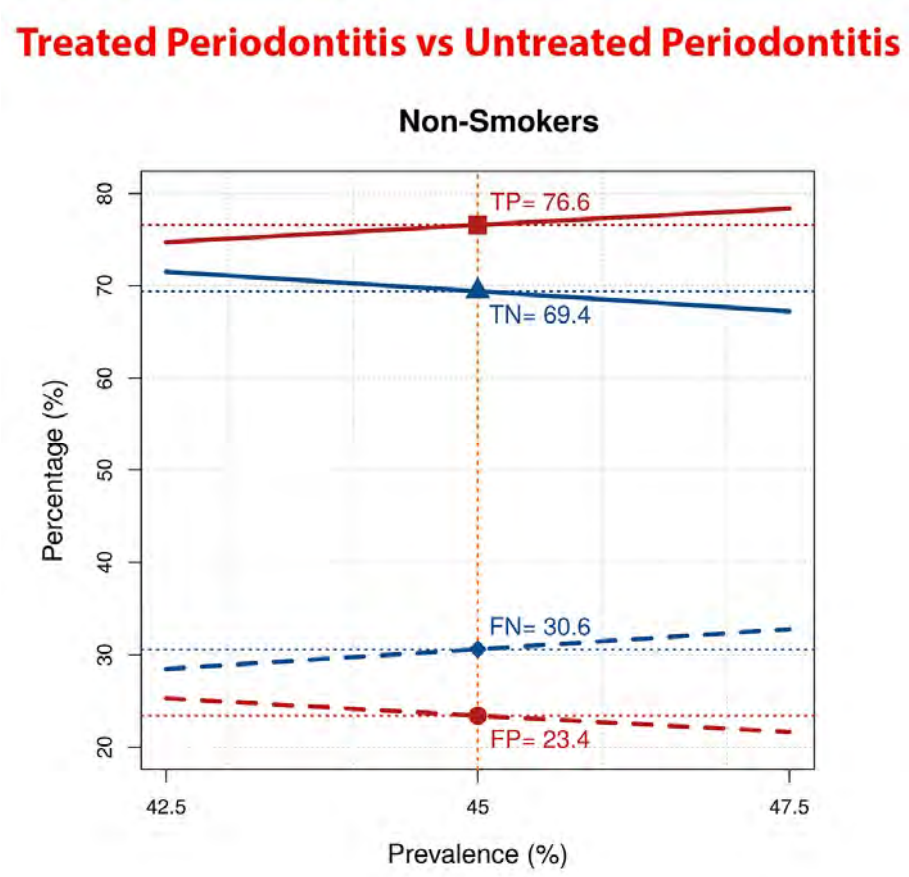

Figure 4C. Predictive percentages of salivary IL1beta biomarker for different prevalence values of periodontitis in non-smokers (second modelling).

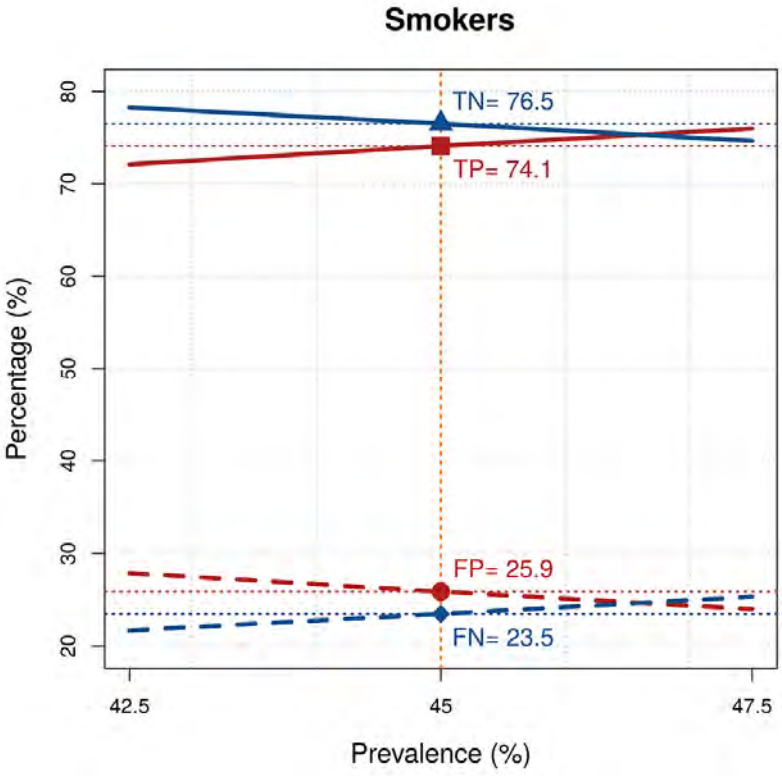

Figure 4D. Predictive percentages of salivary IL1beta biomarker for different prevalence values of periodontitis in smokers (second modelling).

The continuous line indicates the true cases for the different prevalence of periodontitis (the blue line, the true negatives; the red line, the true positives). The discontinuous line indicates false cases for the different prevalence of periodontitis (the blue line, false negatives; the red line, false positives). TP: true positive, test is positive (indicates periodontitis and patient has periodontitis); FP: false positive, test is positive (indicates periodontitis but patient does not have periodontitis); TN: true negative, test is negative (indicates periodontitis not present and patient does not have periodontitis); FN: false negative, test is negative (indicates periodontitis not present but patient has periodontitis).

5.5. DISCUSSION

Ideally, a salivary diagnostic tool would be a non-invasive, complementary test for the diagnosis and monitoring of periodontal patients [6]. It could also be beneficial for the early detection of periodontitis in epidemiological studies [43].

However, if the natural progression of periodontitis substantially complicates the identification of biomarkers in gingival crevicular fluid (GCF) [2], it is reasonable to assume that this problem would be even greater in saliva. Although the advantages associated with the use of salivary samples are evident [6], there are also drawbacks in their manipulation that must be considered [44-46]. The diagnostic accuracy of any salivary biomarker must overcome possible intra- and inter-subject variations due to factors other than the periodontal status [47].

5.5.1. Diagnostic Accuracy of Salivary IL1beta for Detecting Untreated Periodontitis in Non-Smokers and Smokers

After reviewing the literature, we identified research that focused on evaluating whether salivary IL1beta levels can distinguish between patients with different periodontal conditions [14-20]. After analysing these studies in-depth, we verified the heterogeneity concerning methodological factors that may affect the diagnostic classification parameters associated with a biomarker. Unlike previously published articles, the present study calculates predictive models that differentiate between non-smokers and smokers in order to examine, for the first time, the influence of a "smoking habit" on the diagnostic accuracy of salivary IL1beta for detecting untreated periodontitis.

Based on the literature, the capacity of salivary IL1beta to distinguish a non-periodontal subject from one with the disease varies from an AUC value of 0.960 [18] to one of 0.787 [20]. Consequently, the discriminatory potential of IL1beta in the saliva is interpreted as a range from outstanding to acceptable [28]. Our research group has recently demonstrated that the levels of IL1beta in GCF have an outstanding capacity to distinguish patients with periodontitis from those without the condition (AUC values of 0.963) [48]. In our view, it

is relatively surprising that some authors have detected that salivary IL1beta is associated with AUC values ≥0.950 [14,15,18], as these values are similar to those described in the GCF, this being the fluid present in the area where the disease develops [48].

In the three identified studies that determined that IL1beta in saliva had AUC values ≥0.950 [14,15,18], there was a lower number of control patients than periodontal patients in all of them (≤30 individuals). Interestingly, the specificity percentages detected in the three series were very high, and also higher than the sensitivity values [14,15,18], perhaps because of the reduced sample size, directly affecting the discriminatory value of the AUC. Confirming this observation, and following their study in 2013, the research group of Ebersole et al. [16] re-evaluated their sample of more than 100 controls and periodontal patients with similar clinical characteristics two years later. In this second investigation, the salivary IL1beta AUC values were much lower than those initially identified by the authors in their first paper (0.830 *versus* 0.950) [15,16].

In contrast, of the three studies in which IL1beta was described with AUC values ≥ 0.950 [14,15,18], two of them involved only non-smoking patients [14,18], while other authors who detected lower AUC values (≤ 0.800) examined combined groups of non-smokers and smokers [17,20]. This first impression on the possible influence of a "smoking habit" is confirmed by the results of the present study. In our non-smoker series, according to Hosmer et al. [28], the IL1beta salivary levels had an excellent capacity to distinguish untreated periodontitis from periodontal health, although this capability was notably reduced in patients who smoked (AUC values of 0.830 and 0.689, respectively).

Although it has been suggested that IL1beta can discriminate between inactive and active periodontal lesions, there are very few diagnostic accuracy studies that have investigated it in saliva as a way to evaluate the response to periodontal treatment [49,50]. The methodological approach in the present paper is different because we evaluate for the first time the capacity of IL1beta in saliva to distinguish untreated from treated periodontitis, demonstrating clinical

improvement and differentiating between non-smokers and smokers. In this regard, the salivary IL1beta levels maintained an acceptable capacity to discriminate untreated from treated periodontitis, although, curiously, this capability was slightly reduced in non-smokers (AUC values of 0.671 and 0.708 in smokers).

On the other hand, the nomograms derived from our predictive models showed that, for any probability of suffering from periodontitis, the salivary levels of IL1beta that predict this were much higher in non-smokers than in smokers. These findings confirm the immunosuppressive effect associated with tobacco [51] and its impact on IL1beta salivary levels in different clinical conditions. Like earlier results from our group on GCF samples [52], the diagnostic threshold value of IL1beta was higher in non-smokers than in smokers in both modelling phases (periodontal health-untreated periodontitis modelling: 84.76 pg/ml and 42.78 pg/ml; treated periodontitis-untreated periodontitis modelling: 163.50 pg/ml and 27.66 pg/ml). It is interesting to note that in non-smokers the second threshold showed a much higher value than the first threshold, which could indicate that in these patients the practice of periodontal treatment, although reduced to levels of IL1beta saliva, did not reach those present in a periodontal health condition. However, curiously, the opposite situation occurs in smokers: the second threshold showed a much lower value than the first threshold, which could suggest a combined immunosupressive effect of tobacco and periodontal treatment on IL1beta salivary levels.

These findings suggest that, in addition to other influential factors [53], the diagnostic power of the selected diagnostic threshold value is highly dependent on smoking habit.

From a theoretical point of view, an ideal diagnostic test is one that has sensitivity and specificity values close to 100% [54]. However, until now, compliance with this characteristic has been almost impossible when evaluating salivary biomarkers for the diagnosis of periodontitis. In the case of IL1beta, the described sensitivity values ranged from 88%-54% and specificity from 100%-52% [14-20]. This discrepancy may be due to the differences observed between the studies concerning

the different control groups and the different groups of periodontal patients analysed. In the diagnostic accuracy field, it is admitted that the spectrum of clinical conditions has a direct effect on the estimation of sensitivity and specificity [55].

In our study, the sensitivity and specificity values were higher in non-smokers than in smokers in the first modelling phase (72% and 82.5% *versus* 67% and 77%); interestingly, in the second phase, the sensitivity value was higher in smokers (70% *versus* 53% in non-smokers), while the specificity score was higher in non-smokers (87% *versus* 80% in smokers). According to De Luca de Canto et al. [39], these results suggest that IL1beta has a good capability to distinguish periodontal health from untreated periodontitis in non-smokers, but this capacity is only acceptable in smokers; on the other hand, IL1beta had a good capability to distinguish untreated from treated periodontitis in smokers, although its capacity to detect untreated periodontitis was worsened in non-smokers.

5.5.2. Salivary Test for IL1beta in Non-Smokers and Smokers: Implications for Practice

From a clinical point of view, a diagnostic test may be useful or practical depending on the purpose of its application, even if its sensitivity and specificity values are not particularly high [56]. In this sense, it is essential to analyse the predictive values associated with a diagnostic test assessing the prevalence of a disease. Our series is the first on this subject in which this type of analysis is carried out. According to our findings, and assuming a 45% prevalence of periodontitis in terms of its different severity [41,42], if a salivary IL1beta test was applied as a first-line screening tool to determine who should be referred for subsequent periodontal evaluation, of the total tests performed, there would be about 70%-78% of them that would have correctly identified the clinical condition.

Although the findings from the internal validation of the discrimination measures were optimal, the enormous clinical difficulty represented by the recruitment of smokers with periodontal health is a

limitation of this study; equally, reduced sample sizes of groups in the second modelling phase [57]. Consequently, the next objective would be to externally validate the salivary IL1beta-based models obtained in the present series to confirm the universal applicability of our data.

In conclusion, salivary IL1beta has an excellent diagnostic capability for distinguishing systemically healthy patients with untreated periodontitis from those who are periodontally healthy, although this discriminatory potential is reduced in smokers. The diagnostic capacity of salivary IL1beta remains acceptable for discriminating between untreated and treated periodontitis, especially in smokers. The diagnostic threshold values of salivary IL1beta in smokers are lower than in non-smokers in different clinical settings, evidencing the importance of determining the specific diagnostic thresholds of this biomarker in both smoking conditions.

5.6. REFERENCES

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CONCLUSIONS

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Conclusions

1. Derived from the systematic review/meta-analysis of the diagnostic accuracy studies in crevicular gingival fluid, matrix metalloproteinase 8 shows good sensitivity and excellent specificity resulting in the clinically most useful or effective biomarker for a broad spectrum of periodontitis in systemically healthy subjects. Other molecules, such as myeloperoxidase or several pro-inflammatory cytokines, are identified as promising diagnostic biomarkers, but more high-quality research is required to confirm these observations.

2. Derived from the systematic review/meta-analysis of the diagnostic accuracy studies in saliva, matrix metalloproteinases 8 and 9, interleukins 1beta and 6, and haemoglobin are salivary biomarkers with good capability to detect periodontitis in systemically healthy subjects; matrix metalloproteinase 9 and interleukin 1beta also show good capability to detect the non-periodontitis condition. Matrix metalloproteinase 8 and interleukin 1beta are the most researched salivary biomarkers in the diagnostic accuracy field, both presenting clinically fair effectiveness for the diagnosis of a broad spectrum of periodontitis. Other molecules, such as cysteine, macrophage inflammatory protein 1alpha and nitric oxide (and its related-metabolites), are identified as promising salivary biomarkers, but more high-quality research is needed to confirm these observations.

3. Interleukin 1alpha, interleukin 1beta and interleukin 17A in the gingival crevicular fluid are outstanding biomarkers for distinguishing systemically healthy patients with chronic periodontitis from periodontally healthy individuals. The predictive ability of these pro-inflammatory cytokines is increased by incorporating interferon gamma and interleukin 10. In the nomograms, higher levels of these

pro-inflammatory cytokines and being a smoker increase the probability of having chronic periodontitis (potentiating role), while interferon gamma and interleukin 10 have the opposite function (protective role). The clinical implications of these biomarkers could include improved patient monitoring and the control of disease activity, although external validation studies are needed to confirm the universal applicability of our findings.

4. Interleukins 1alpha, 1beta and 17A, and their ratios with interleukin 2 are excellent biomarkers in gingival crevicular fluid for distinguishing systemically healthy subjects with chronic periodontitis from periodontally healthy individuals, independently of smoking status. Cytokine thresholds in GCF with diagnostic potential are defined, showing that smokers have lower threshold values than non-smokers. This fact reveals the convenience of designing GCF biomarker studies for predicting periodontitis differentiating by smoking status, especially if the diagnostic thresholds are to be accurately defined.

5. Interleukin 1beta in the saliva is an excellent biomarker for distinguishing systemically healthy patients with untreated periodontitis from those who are periodontally healthy, although this discriminatory potential is reduced in smokers. The diagnostic capacity of this salivary cytokine remains acceptable for discriminating between untreated and treated periodontitis patients, especially in smokers. The diagnostic threshold values of salivary interleukin 1beta in smokers are lower than in non-smokers in different clinical settings, evidencing the importance of determining the specific diagnostic thresholds of this salivary biomarker in both smoking conditions.

APPENDICES

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Appendices Introduction

Titles of the Appendices

Appendix S1. Checklist for recommendations of Thesis EDI saúde (general).

Appendix S2. Permissions, which were given by the publishers for the use of non-original figures in the Introduction of the present Thesis.

APPENDIX S1

Yes-No-N/A		Page
FOR ALL THESIS		
Yes	Declaration of potential conflicts of interests	188,258,320,372,404
Yes	Declaration on the origin and copyright status of non-original figures, with permission if necessary. Include them in the text of each figure*	58,59,85,88,92,96,97,99, 103,107,109,112, 115,118,121,122
Yes	Checklist of statistics adequacy if no other checklists apply**	450-452,479-481 (PRISMA*);513-515, 529-531,537-539 (TRIPOD**)
FOR THESIS INVOLVING HUMAN SAMPLES, OR PERSONAL DATA		
Yes	Declaration on approval by the research ethics committee	325,375-376,408
Yes	Code number of the study	2015/006
Yes	Copy of ethics report	Separate PDF reported
N/A	Declaration that data are based on anonymous information, and no approval of the ethics committee is needed	-
N/A	If it is an observational study, STROBE checklist**	513-515,529-531,537-539 (TRIPOD**)
FOR THESIS THAT INCLUDES A CLINICAL ASSAY		
N/A	Declaration of its authorisation by the Agencia Española de Medicamentos y productos sanitarios	-
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FOR THESIS THAT INCLUDES ANIMAL EXPERIMENTATION		
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N/A	Code number of the authorization of the animal experimentation Project.	-
N/A	Register number of the authorised user centre if experiments were made in Spain	-
N/A	Copy of the capacitation certificate if the experiments were made by the Thesis author	-
N/A	Person, company or service that performed the experiments if applicable	-
N/A	ARRIVE Checklist	-

*Figures that do not show a text on the declaration of origin are original figures of own design

**The PRISMA checklist is the recommended checklist in systematic reviews and meta-analyses of diagnostic precision studies

**The TRIPOD checklist is the recommended checklist in diagnostic accuracy studies in which predictive modelling techniques are applied

PhD Student Signature

APPENDIX S2

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Appendices Objective 1

Accuracy of Single Molecular Biomarkers in Gingival Crevicular Fluid for the Diagnosis of Periodontitis in Systemically Healthy Subjects: A Systematic Review and Meta-Analysis.

Titles of Appendices

Appendix S1. PRISMA-DTA checklist.

Appendix S2. Articles excluded from this systematic review, detailing the reasons for exclusion (n=108 articles).

Appendix S3. Sample sizes of the 2x2 contingency tables of the diagnostic classifications of MMP8, cathepsin, trypsin and elastase included in the meta-analysis, detailing the sensitivity and specificity values obtained within-classification and between-classifications applying the HSROC modelling.

Appendix S4. Meta-analyses performed on the MMP8 biomarker using HSROC modelling following different criteria: selection of the contingency tables according to the best classification parameters (S4.1, upper graph); selection of the contingency tables based on healthy patients *versus* periodontitis patients (S4.1, lower graph); selection of the contingency tables in which MMP8 was determined by ELISA (S4.2, upper graph); selection of the contingency tables in which MMP8 was determined by IFMA (S4.2, lower graph).

APPENDIX S1

Section/Topic	#	PRISMA-DTA Checklist Item	Reported on Page
TITLE/ABSTRACT			
Title	1	Identify the report as a systematic review (+/- meta-analysis) of diagnostic test accuracy (DTA) studies	187
Abstract	2	Abstract: See PRISMA-DTA for abstracts	187,188
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known	189-192
Clinical role of index test	D1	State the scientific and clinical background, including the intended use and clinical role of the index test, and if applicable, the rationale for minimally acceptable test accuracy (or minimum difference in accuracy for comparative design)	189-192
Objectives	4	Provide an explicit statement of question(s) being addressed in terms of participants, index test(s), and target condition(s)	192,193
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number	192
Eligibility criteria	6	Specify study characteristics (participants, setting, index test(s), reference standard(s), target condition(s), and study design) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale	193-195
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched	195
Search	8	Present full search strategies for all electronic databases and other sources searched, including any limits used, such that they could be repeated	195-199 Table 1 Table 2
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis)	197-199
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators	200
Definitions for data extraction	11	Provide definitions used in data extraction and classifications of target condition(s), index test(s), reference standard(s) and other characteristics (e.g. study design, clinical setting)	200

Risk of bias and applicability	12	Describe methods used for assessing risk of bias in individual studies and concerns regarding the applicability to the review question	200-202 Table 3
Diagnostic accuracy measures	13	State the principal diagnostic accuracy measure(s) reported (e.g. sensitivity, specificity) and state the unit of assessment (e.g. per-patient, per-lesion)	202-204 Table 4
Synthesis of results	14	Describe methods of handling data, combining results of studies and describing variability between studies. This could include, but is not limited to: a) handling of multiple definitions of target condition. b) handling of multiple thresholds of test positivity, c) handling multiple index test readers, d) handling of indeterminate test results, e) grouping and comparing tests, f) handling of different reference standards	202-204
Meta-analysis	D2	Report the statistical methods used for meta-analyses, if performed	204,205
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified	205,206
RESULTS			
Study selection	17	Provide numbers of studies screened, assessed for eligibility, included in the review (and included in meta-analysis, if applicable) with reasons for exclusions at each stage, ideally with a flow diagram	206, Figure 1, Appendix S2
Study characteristics	18	For each included study provide citations and present key characteristics including: a) participant characteristics (presentation, prior testing), b) clinical setting, c) study design, d) target condition definition, e) index test, f) reference standard, g) sample size, h) funding sources	208, Table 5
Risk of bias and applicability	19	Present evaluation of risk of bias and concerns regarding applicability for each study	212, Figure 2
Results of individual studies	20	For each analysis in each study (e.g. unique combination of index test, reference standard, and positivity threshold) report 2x2 data (TP, FP, FN, TN) with estimates of diagnostic accuracy and confidence intervals, ideally with a forest or receiver operator characteristic (ROC) plot	213, 223, Figures 3, Table 6, Figures 6, Table 7
Synthesis of results	21	Describe test accuracy, including variability; if meta-analysis was done, include results and confidence intervals	218, Figures 4, Appendix S3, Appendix S4
Additional analysis	22	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression; analysis of index test: failure rates, proportion of inconclusive results, adverse events)	223, Figures 5
DISCUSSION			

Summary of evidence	23	Summarize the main findings including the strength of evidence	232-238
Limitations	24	Discuss limitations from included studies (e.g. risk of bias and concerns regarding applicability) and from the review process (e.g. incomplete retrieval of identified research)	240-241
Conclusions	25	Provide a general interpretation of the results in the context of other evidence. Discuss implications for future research and clinical practice (e.g. the intended use and clinical role of the index test)	239-242
FUNDING			
Funding	26	For the systematic review, describe the sources of funding and other support and the role of the funders	188

APPENDIX S2

Author	Reason for Exclusion	Author	Reason for Exclusion
Akbari et al. [1]	1	Hanioka et al. [36]	9
Alpagot et al. [2]	2	Harb et al. [37]	2
Alpagot et al. [3]	2	Hardan et al. [38]	2
Bader & Boyd [4]	7	Imamura et al. [39]	3
Baliban et al. [5]	2	Inomata et al. [40]	2
Baliban et al. [6]	9	Ishisaka et al. [41]	8
Beighton et al. [7]	9	Ito et al. [42]	2
Beighton et al. [8]	9	Johannsen et al. [43]	2
Booth et al. [9]	2	Kajiura et al. [44]	6
Boyer et al. [10]	3	Kakuta et al. [45]	3
Chaparro et al. [11]	1	Kaman et al. [46]	2
Cooke et al. [12]	8	Kaner et al. [47]	7
Corbi et al. [13]	5	Kaslick et al. [48]	2
Cox & Eley [14]	2	Kim et al. [49]	1
Cox et al. [15]	2	Kinney et al. [50]	9
Dabiri et al. [16]	2	Kobayashi et al. [51]	5
Dongari-Bagtzoglou et al. [17]	2	Kobayashi et al. [52]	5
Dutzan et al. [18]	2	Kraft-Neumärker et al. [53]	2
Ebersole et al. [19]	2	Krasse & Egelberg [54]	2
Eick et al. [20]	3	Kurihara et al. [55]	1
Eley & Cox [21]	7	Lamster et al. [56]	2
Eley & Cox [22]	7	Lamster et al. [57]	2
Eley & Cox [23]	7	Lamster et al. [58]	7
Eley & Cox [24]	7	Lamster et al. [59]	2
Feng et al. [25]	1	Lamster et al. [60]	7
Fine et al. [26]	7	Lappin et al. [61]	2
Fitzsimmons et al. [27]	2	Lee et al. [62]	2
Friedman et al. [28]	2	Leppilahti et al. [63]	2
Galassi et al. [29]	4	Leppilahti et al. [64]	2
Genco et al. [30]	8	Levine et al. [65]	8
Genco et al. [31]	1	Loesche et al. [66]	2
Giannobile et al. [32]	5	Machtei et al. [67]	1
Gleissner et al. [33]	6	Magnusson et al. [68]	2
Grbic et al. [34]	2	Mancini et al. [69]	2
Guzman et al. [35]	2	Moss et al. [70]	1

Author	Reason for Exclusion	Author	Reason for Exclusion
Ngo et al. [71]	10	Schmickler et al. [90]	1
Niekrash & Patters [72]	2	Shaimaa et al. [91]	6
Oliver et al. [73]	1	Shimada et al. [92]	1
Oringer et al. [74]	7	Sims et al. [93]	2
Palcanis et al. [75]	7	Snyder et al. [94]	3
Paolantonio et al. [76]	4	Strauss et al. [95]	1
Pereira et al. [77]	3	Surna et al. [96]	2
Persson et al. [78]	2	Tanner et al. [97]	3
Persson et al. [79]	2	Taubman et al. [98]	2
Rakmanee et al. [80]	8	Teles et al. [99]	2
Rams et al. [81]	3	Tsuchida et al. [100]	2
Reddy et al. [82]	2	Vienneau & Kindberg [101]	2
Reit et al. [83]	3	Wignarajah et al. [102]	2
Rühling et al. [84]	4	Xiang et al. [103]	9
Salih [85]	2	Xiang et al. [104]	1
Sánchez-Pérez et al. [86]	4	Yamamoto et al. [105]	5
Sanders et al. [87]	2	Yoshinari et al. [106]	2
Sarment et al. [88]	2	Zani et al. [107]	4
Schacher et al. [89]	3	Zheng et al. [108]	2

REASONS FOR EXCLUSION

(1) Patients with an explicit diagnosis of systemic disease, alcoholism or pregnancy condition (n=13);
(2) Not reported sensitivity and specificity values for diagnosis of periodontitis nor sufficient data to calculate the 2 x 2 contingency table (n=49);
(3) Salivary biomarkers based on bacteria (n=10);
(4) Target conditions other than periodontitis (asymptomatic apical periodontitis or peri-implantitis) (n=5);
(5) Genetic or animal studies (n=5);
(6) Lack of definition of clinical parameters used for diagnosis or standard reference based in gingival inflammation parameter (n=3);
(7) Salivary biomarkers used to predict the periodontitis progression or treatment response (n=11);
(8) Different biological media (n=5);
(9) Accuracy studies on multi-biomarker models (n=6);
(10) Accuracy studies on biomarkers not defined (n=1).

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APPENDIX S3

Appendix S3.1

SAMPLE SIZE (MMP8)					
	Total	+ +	+ -	- +	- -
Classification 1 Baeza et al. [50]	62	30	3	1	28
Classification 2 Baeza et al. [50]	62	28	6	3	25
Classification 3 Mäntylä et al. [55]	149	58	1	32	58
Classification 4 Mäntylä et al. [55]	148	58	9	32	49
Classification 5 Mäntylä et al. [55]	207	58	10	32	107
Classification 6 Mäntylä et al. [55]	149	63	1	27	58
Classification 7 Mäntylä et al. [55]	148	63	13	27	45
Classification 8 Mäntylä et al. [55]	207	63	14	27	103
Classification 9 Leppilahti et al. [51]	58	18	2	1	37
Classification 10 Leppilahti et al. [51]	38	17	2	2	17
Classification 11 Gul et al. [53]	231	132	13	22	64
Classification 12 Gul et al. [52]	90	54	3	6	27
Classification 13 Yuan et al. [54]	358	142	18	36	162
WITHIN-CLASSIFICATION SENSITIVITY (MMP8)					
	Estimate	Standard Dev.	MC Error	CI lower	CI upper
Classification 1 Baeza et al. [50]	0.917	0.046	0.001	0.822	0.990
Classification 2 Baeza et al. [50]	0.877	0.050	0.001	0.775	0.966
Classification 3 Mäntylä et al. [55]	0.655	0.048	0.001	0.558	0.749
Classification 4 Mäntylä et al. [55]	0.666	0.047	0.001	0.573	0.757
Classification 5 Mäntylä et al. [55]	0.661	0.048	0.001	0.563	0.752
Classification 6 Mäntylä et al. [55]	0.706	0.0462	0.001	0.612	0.793
Classification 7 Mäntylä et al. [55]	0.717	0.045	0.001	0.623	0.801
Classification 8 Mäntylä et al. [55]	0.712	0.044	0.001	0.623	0.796
Classification 9 Leppilahti et al. [51]	0.882	0.061	0.001	0.754	0.980

Classification 10 Leppilahti et al. [51]	0.856	0.064	0.001	0.721	0.963
Classification 11 Gul et al. [53]	0.853	0.027	0.000	0.798	0.905
Classification 12 Gul et al. [52]	0.882	0.038	0.000	0.802	0.951
Classification 13 Yuan et al. [54]	0.795	0.029	0.001	0.734	0.850
WITHIN-CLASSIFICATION SPECIFICITY (MMP8)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Classification 1 Baeza et al. [50]	0.892	0.045	0.000	0.799	0.969
Classification 2 Baeza et al. [50]	0.847	0.050	0.001	0.738	0.930
Classification 3 Mäntylä et al. [55]	0.955	0.024	0.001	0.905	0.995
Classification 4 Mäntylä et al. [55]	0.868	0.039	0.002	0.783	0.933
Classification 5 Mäntylä et al. [55]	0.914	0.023	0.001	0.865	0.954
Classification 6 Mäntylä et al. [55]	0.950	0.024	0.001	0.903	0.993
Classification 7 Mäntylä et al. [55]	0.821	0.047	0.002	0.724	0.903
Classification 8 Mäntylä et al. [55]	0.886	0.026	0.001	0.833	0.934
Classification 9 Leppilahti et al. [51]	0.921	0.036	8e-04	0.846	0.981
Classification 10 Leppilahti et al. [51]	0.894	0.048	0.000	0.788	0.969
Classification 11 Gul et al. [53]	0.850	0.035	0.001	0.776	0.914
Classification 12 Gul et al. [52]	0.891	0.042	0.001	0.804	0.964
Classification 13 Yuan et al. [54]	0.900	0.021	0.001	0.856	0.939
BETWEEN-CLASSIFICATIONS SENSITIVITY AND SPECIFICITY (MMP8)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Sensitivity	0.758	0.136	0.003	0.505	1
Specificity	0.929	0.083	0.003	0.747	0.999

Appendix S3.2

SAMPLE SIZE (ELASTASE)					
	Total	+ +	+ -	- +	- -
Classification 1 Gul et al. [53]	231	120	15	34	62
Classification 2 Gul et al. [52]	90	53	3	7	27
Classification 3 Ito et al. [58]	283	41	21	37	184
Classification 4 Eley & Cox [56]	528	192	112	48	176
Classification 5 Eley & Cox [57]	528	235	69	55	169
WITHIN-CLASSIFICATION SENSITIVITY (ELASTASE)					
	Estimate	Standard Dev.	MC Error	CI lower	CI upper
Classification 1 Gul et al. [53]	0.776	0.032	0.001	0.712	0.839
Classification 2 Gul et al. [52]	0.864	0.045	0.001	0.770	0.944
Classification 3 Ito et al. [58]	0.543	0.055	0.002	0.439	0.655
Classification 4 Eley & Cox [56]	0.799	0.025	0.001	0.749	0.848
Classification 5 Eley & Cox [57]	0.809	0.022	0.000	0.765	0.851
WITHIN-CLASSIFICATION SPECIFICITY (ELASTASE)					
	Estimate	Standard Dev.	MC Error	CI lower	CI upper
Classification 1 Gul et al. [53]	0.803	0.042	0.002	0.720	0.885
Classification 2 Gul et al. [52]	0.852	0.065	0.002	0.719	0.962
Classification 3 Ito et al. [58]	0.893	0.021	0.001	0.848	0.931
Classification 4 Eley & Cox [56]	0.622	0.028	0.001	0.566	0.680
Classification 5 Eley & Cox [57]	0.715	0.029	0.001	0.655	0.769
BETWEEN-CLASSIFICATIONS SENSITIVITY AND SPECIFICITY (ELASTASE)					
	Estimate	Standard Dev.	MC Error	CI lower	CI upper
Sensitivity	0.746	0.208	0.004	0.294	1
Specificity	0.811	0.192	0.004	0.380	1

Appendix S3.3

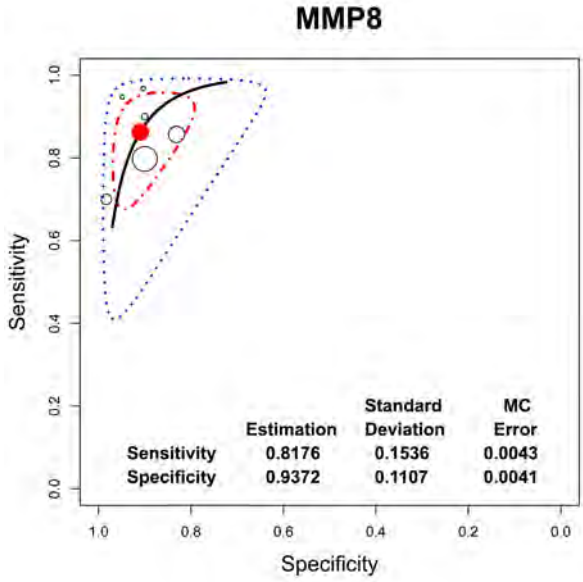
SAMPLE SIZE (CATHEPSIN)					
	Total	**+ +**	**+ -**	**- +**	**- -**
Classification 1 Gul et al. [52]	90	33	15	27	15
Classification 2 Eley & Cox [57]	528	192	101	48	187
Classification 3 Eley & Cox [56]	528	244	48	46	190
WITHIN-CLASSIFICATION SENSITIVITY (CATHEPSIN)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Classification 1 Gul et al. [52]	0.569	0.062	0.002	0.451	0.697
Classification 2 Eley & Cox [57]	0.794	0.026	0.001	0.744	0.846
Classification 3 Eley & Cox [56]	0.842	0.021	0.000	0.799	0.882
WITHIN-CLASSIFICATION SPECIFICITY (CATHEPSIN)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Classification 1 Gul et al. [52]	0.513	0.081	0.004	0.351	0.672
Classification 2 Eley & Cox [57]	0.652	0.028	0.001	0.597	0.708
Classification 3 Eley &Cox [56]	0.792	0.027	0.001	0.739	0.845
BETWEEN-CLASSIFICATIONS SENSITIVITY AND SPECIFICITY (CATHEPSIN)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Sensitivity	0.728	0.235	0.005	0.200	1
Specificity	0.673	0.242	0.004	0.163	1

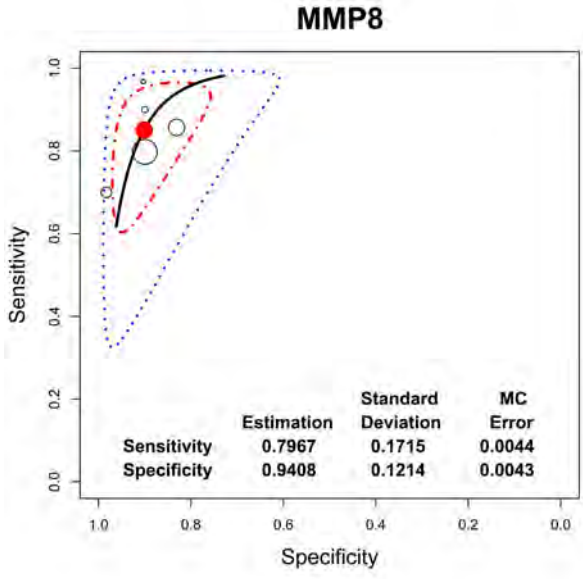
Appendix S3.4

SAMPLE SIZE (TRYPSIN)					
	Total	**+ +**	**+ -**	**- +**	**- -**
Classification 1 Gul et al. [52]	90	48	13	12	17
Classification 2 Eley & Cox [57]	528	178	107	62	181
Classification 4 Eley & Cox [56]	528	206	76	84	162
WITHIN-CLASSIFICATION SENSITIVITY (TRYPSIN)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Classification 1 Gul et al. [52]	0.783	0.047	0.001	0.692	0.876
Classification 2 Eley & Cox [57]	0.742	0.026	0.001	0.690	0.794
Classification 4 Eley & Cox [56]	0.709	0.025	0.001	0.658	0.758
WITHIN-CLASSIFICATION SPECIFICITY (TRYPSIN)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Classification 1 Gul et al. [52]	0.605	0.064	0.002	0.469	0.723
Classification 2 Eley & Cox [57]	0.634	0.026	0.001	0.581	0.685
Classification 4 Eley & Cox [56]	0.674	0.027	0.001	0.620	0.729
BETWEEN-CLASSIFICATIONS SENSITIVITY AND SPECIFICITY (TRYPSIN)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Sensitivity	0.713	0.175	0.004	0.342	1
Specificity	0.661	0.180	0.003	0.293	1

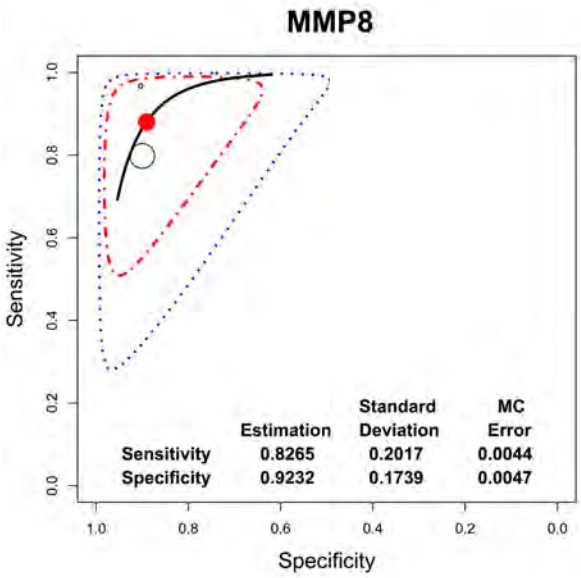
APPENDIX S4

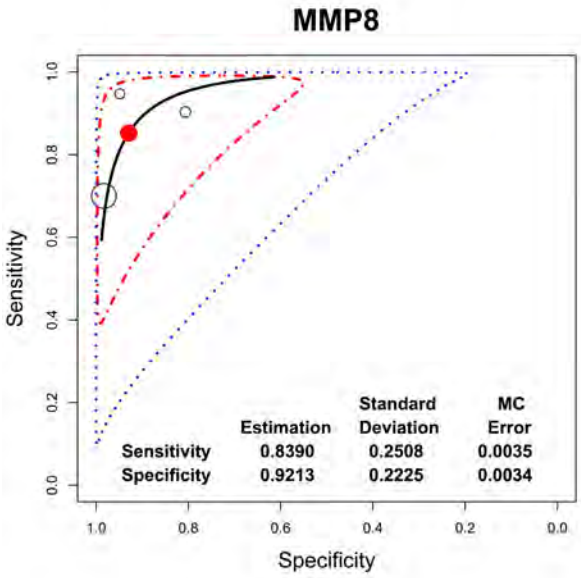
Appendix S4.1





Appendix S4.2





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Appendices Objective 2

How Accurate Are Single Molecular Biomarkers in Saliva for the Diagnosis of Periodontitis? A Systematic Review and Meta-Analysis.

Titles of Appendices

Appendix S1. PRISMA-DTA checklist.

Appendix S2. Articles excluded from this systematic review, detailing the reasons for exclusion (n=92 articles).

Appendix S3. Sample sizes of the 2x2 contingency tables of the diagnostic classifications of MMP8, MMP9, IL1beta, IL6 and Haemoglobin included in the meta-analysis, detailing the sensitivity and specificity values obtained within-classification and between-classifications applying the HSROC modelling.

Appendix S4. Meta-analyses performed on the MMP8 biomarker using HSROC modelling following different criteria: selection of the contingency tables according to the best classification parameters (S4.1, upper graph); selection of the contingency tables based on healthy patients *vs* periodontitis patients (S4.1, lower graph); selection of the contingency tables based on healthy patients and with gingivitis *vs* periodontitis patients (S4.2, upper graph); selection of the contingency tables in which MMP8 was determined by ELISA (S4.2, lower graph).

Appendix S5. Meta-analyses performed on the MMP9 biomarker using HSROC modelling following different criteria: selection of the contingency tables according to the best classification parameters.

Appendix S6. Meta-analyses performed on the IL1beta biomarker using HSROC modelling following different criteria: selection of the contingency tables based on healthy patients *versus* periodontitis patients (S6.1, upper graph); selection of the contingency tables based on healthy patients and with gingivitis *versus* periodontitis patients (S6.1, lower graph); selection of the contingency tables in which IL1beta was determined by ELISA (S6.2, upper graph); selection of the contingency tables in which IL1beta was determined by multiparametric cytometry (S6.2, lower graph).

Appendix S7. Meta-analyses performed on the IL6 biomarker using HSROC modelling following different criteria: selection of the contingency tables based on healthy patients and with gingivitis *versus* periodontitis patients (upper graph); selection of the contingency tables in which IL6 was determined by multiparametric cytometry (lower graph).

Appendix S8. Meta-analyses performed on the Hb biomarker using HSROC modelling following different criteria: selection of the contingency tables according to the best classification parameters.

APPENDIX S1

Section/ Topic	#	PRISMA-DTA Checklist Item	Reported on Page #
TITLE/ABSTRACT			
Title	1	Identify the report as a systematic review (+/- meta-analysis) of diagnostic test accuracy (DTA) studies	257
Abstract	2	Abstract: See PRISMA-DTA for abstracts	257, 258
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known	259,260
Clinical role of index test	D1	State the scientific and clinical background, including the intended use and clinical role of the index test, and if applicable, the rationale for minimally acceptable test accuracy (or minimum difference in accuracy for comparative design)	259,260
Objectives	4	Provide an explicit statement of question(s) being addressed in terms of participants, index test(s), and target condition(s)	260,261
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number	261
Eligibility criteria	6	Specify study characteristics (participants, setting, index test(s), reference standard(s), target condition(s), and study design) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale	261-263
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched	263
Search	8	Present full search strategies for all electronic databases and other sources searched, including any limits used, such that they could be repeated	263-266 Table 1
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis)	265-266
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators	266
Definitions for data extraction	11	Provide definitions used in data extraction and classifications of target condition(s), index test(s), reference standard(s) and other characteristics (e.g. study design, clinical setting)	266

Risk of bias and applicability	12	Describe methods used for assessing risk of bias in individual studies and concerns regarding the applicability to the review question	267
Diagnostic accuracy measures	13	State the principal diagnostic accuracy measure(s) reported (e.g. sensitivity, specificity) and state the unit of assessment (e.g. per-patient, per-lesion)	267,268
Synthesis of results	14	Describe methods of handling data, combining results of studies and describing variability between studies. This could include, but is not limited to: a) handling of multiple definitions of target condition. b) handling of multiple thresholds of test positivity, c) handling multiple index test readers, d) handling of indeterminate test results, e) grouping and comparing tests, f) handling of different reference standards	267, 268
Meta-analysis	D2	Report the statistical methods used for meta-analyses, if performed	268
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified	268, 269
RESULTS			
Study selection	17	Provide numbers of studies screened, assessed for eligibility, included in the review (and included in meta-analysis, if applicable) with reasons for exclusions at each stage, ideally with a flow diagram	269, Figure 1, Appendix S2
Study characteristics	18	For each included study provide citations and present key characteristics including: a) participant characteristics (presentation, prior testing), b) clinical setting, c) study design, d) target condition definition, e) index test, f) reference standard, g) sample size, h) funding sources	271, Table 2
Risk of bias and applicability	19	Present evaluation of risk of bias and concerns regarding applicability for each study	275, Figure 2
Results of individual studies	20	For each analysis in each study (e.g. unique combination of index test, reference standard, and positivity threshold) report 2x2 data (TP, FP, FN, TN) with estimates of diagnostic accuracy and confidence intervals, ideally with a forest or receiver operator characteristic (ROC) plot	276, Figures 3, Table 3, 288, Figures 6, Table 4
Synthesis of results	21	Describe test accuracy, including variability; if meta-analysis was done, include results and confidence intervals	283, Figures 4, Appendix S3, Appendix S4
Additional analysis	22	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression; analysis of index test: failure rates, proportion of inconclusive results, adverse events)	286, Figures 5
DISCUSSION			

Summary of evidence	23	Summarize the main findings including the strength of evidence	298-307
Limitations	24	Discuss limitations from included studies (e.g. risk of bias and concerns regarding applicability) and from the review process (e.g. incomplete retrieval of identified research)	306
Conclusions	25	Provide a general interpretation of the results in the context of other evidence. Discuss implications for future research and clinical practice (e.g. the intended use and clinical role of the index test)	305-307
FUNDING			
Funding	26	For the systematic review, describe the sources of funding and other support and the role of the funders	258

APPENDIX S2

Author	Reason for Exclusion	Author	Reason for Exclusion
Acharya et al. [1]	4	Karjalainen et al. [36]	2
Adachi et al. [2]	1	Kibayashi et al. [37]	2
Aimetii et al. [3]	1	Kilbourne et al. [38]	1
Akbari et al. [4]	2	Kim et al. [39]	1
Al-Rawi & Shahid [5]	1	Kinney et al. [40]	3
Baehni & Guggenheim [6]	3	Kinney et al. [41]	9
Barbosa et al. [7]	2	Kobayashi et al. [42]	1
Baroni et al. [8]	6	Kosaka et al. [43]	1
Baskaradoss et al. [9]	1	Kuboniwa et al. [44]	2
Benedetti et al. [10]	2	Kugahara et al. [45]	1
Bostanci et al. [11]	2	Kurgan et al. [46]	2
Chaparro et al. [12]	1	Lee et al. [47]	4
Christodoulides et al. [13]	2	Lee et al. [48]	7
Ekuni et al. [14]	4	Leppilahti et al. [49]	2
Feng et al. [15]	1	Leppilahti et al. [50]	1
Ferrando et al. [16]	9	Liljestrand et al. [51]	3
Fine et al. [17]	7	Lorenz et al. [52]	2
Frodge et al. [18]	2	Machtei et al. [53]	1
Gursoy et al. [19]	2	Mäntilä et al. [54]	1
Gursoy et al. [20]	2	Miyoshi et al. [55]	1
Gursoy et al. [21]	2	Morelli et al. [56]	5
Gursoy et al. [22]	2	Morozumi et al. [57]	3
Haririan et al. [23]	2	Nabet et al. [58]	2
Haro et al. [24]	8	Nagao et al. [59]	1
Heikkinen et al. [26]	2	Nagarajan et al. [60]	9
Heikkinen et al. [27]	2	Nagarajan et al. [61]	2
Heikkinen et al. [25]	5	Nam et al. [62]	1
Hilgert et al. [28]	2	Nishida et al. [63]	2
Hirotomi et al. [29]	2	Nomura et al. [64]	2
Huang et al. [30]	2	Nomura et al. [65]	7
Hugo et al. [31]	2	Nwhator et al. [66]	10
Isaza-Guzman et al. [32]	2	Ochanji et al. [67]	9
Ishii et al. [33]	3	Rajesh et al. [68]	2
Ishisaka et al. [34]	2	Redman et al. [69]	1
Kaczor-Urbanowicz et al. [35]	5	Refulio et al. [70]	2

Author	Reason for exclusion	Author	Reason for exclusion
Rzeznik et al. [71]	9	Sugimoto et al. [82]	6
Salih [72]	2	Surna et al. [83]	2
Salminen et al. [73]	3	Tamaki et al. [84]	2
Saygun et al. [74]	3	Tobón-Arroyave et al. [85]	2
Schmidt et al. [75]	2	Villa-Correa et al. [86]	2
Sexton et al. [76]	2	Villa-Correa et al. [87]	2
Shan et al. [77]	2	Waszkiewicz et al. [88]	2
Shi et al. [78]	2	Waszkiewicz et al. [89]	1
Shyu et al. [79]	7	Waszkiewicz et al. [90]	1
Simsek Ozek et al. [80]	9	Yamamoto et al.[91]	2
Singh et al. [81]	2	Yuan et al. [92]	2

REASONS FOR EXCLUSION

(1) Patients with an explicit diagnosis of systemic disease, alcoholism or pregnancy condition (n=20);
(2) Not reported sensitivity and specificity values for diagnosis of periodontitis nor sufficient data to calculate the 2 x 2 contingency table (n=45);
(3) Salivary biomarkers based on bacteria (n=7);
(4) Target conditions other than periodontitis (gingivitis, peri-implantitis, or mixed groups of gingivitis plus periodontitis) (n=3);
(5) Genetic or epidemiological studies (n=3);
(6) Lack of definition of clinical parameters used for diagnosis (n=2);
(7) Salivary biomarkers used to predict the periodontitis progression or treatment response (n=4);
(8) Different biological media (n=1);
(9) Accuracy studies on multi-biomarker models (n=6);
(10) Control conditions other than periodontal health or gingivitis (n=1).

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APPENDIX S3

Appendix S3.1

SAMPLE SIZE (MMP8)					
	Total	+ +	+ -	- +	- -
Classification 1 Ramseier et al. [40]	79	27	12	12	28
Classification 2 Ebersole et al. [37]	80	40	4	10	26
Classification 3 Johnson et al. [35]	41	28	2	3	8
Classification 4 Johnson et al. [35]	102	65	9	7	21
Classification 5 Johnson et al. [35]	143	90	9	23	21
Classification 6 Johnson et al. [35]	41	28	3	3	7
Classification 7 Johnson et al. [35]	41	28	3	3	7
Classification 8 Wu et al. [36]	57	26	14	4	13
Classification 9 Ebersole et al. [38]	209	66	36	35	72
Classification 10 Gursoy et al. [39]	117	20	28	16	53
Classification 11 Gursoy et al. [39]	110	21	28	8	53
Classification 12 Gursoy et al. [39]	165	56	28	28	53
Classification 13 Gursoy et al. [39]	230	97	28	52	53
Classification 14 Izadi et al. [34]	60	26	12	4	18
Classification 15 Izadi et al. [34]	45	14	12	1	18
Classification 16 Izadi et al. [34]	45	12	12	3	18
WITHIN-CLASSIFICATION SENSITIVITY (MMP8)					
	Estimate	Standard Dev.	MC Error	CI lower	CI upper
Classification 1 Ramseier et al. [40]	0.727	0.058	0.001	0.605	0.832
Classification 2 Ebersole et al. [37]	0.796	0.050	0.001	0.691	0.887
Classification 3 Johnson et al. [35]	0.853	0.051	0.000	0.752	0.945
Classification 4 Johnson et al. [35]	0.872	0.035	0.000	0.799	0.937
Classification 5 Johnson et al. [35]	0.793	0.034	0.000	0.721	0.856
Classification 6 Johnson et al. [35]	0.852	0.050	0.000	0.752	0.946

Classification 7 Johnson et al. [35]	0.852	0.050	0.000	0.753	0.945
Classification 8 Wu et al. [36]	0.828	0.057	0.002	0.711	0.929
Classification 9 Ebersole et al. [38]	0.682	0.042	0.001	0.596	0.760
Classification 10 Gursoy et al. [39]	0.648	0.066	0.002	0.512	0.770
Classification 11 Gursoy et al. [39]	0.748	0.061	0.001	0.625	0.864
Classification 12 Gursoy et al. [39]	0.695	0.044	0.001	0.604	0.777
Classification 13 Gursoy et al. [39]	0.670	0.036	0.001	0.595	0.738
Classification 14 Izadi et al. [34]	0.828	0.053	0.001	0.720	0.925
Classification 15 Izadi et al. [34]	0.839	0.063	0.001	0.714	0.954
Classification 16 Izadi et al. [34]	0.791	0.068	0.001	0.649	0.912
WITHIN-CLASSIFICATION SPECIFICITY (MMP8)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Classification 1 Ramseier et al. [40]	0.677	0.048	0.001	0.581	0.774
Classification 2 Ebersole et al. [37]	0.726	0.056	0.002	0.619	0.840
Classification 3 Johnson et al. [35]	0.686	0.072	0.002	0.543	0.822
Classification 4 Johnson et al. [35]	0.676	0.066	0.003	0.537	0.792
Classification 5 Johnson et al. [35]	0.676	0.051	0.002	0.573	0.776
Classification 6 Johnson et al. [35]	0.671	0.071	0.002	0.530	0.806
Classification 7 Johnson et al. [35]	0.670	0.072	0.002	0.522	0.807
Classification 8 Wu et al. [36]	0.607	0.066	0.003	0.471	0.726
Classification 9 Ebersole et al. [38]	0.662	0.038	0.001	0.586	0.734
Classification 10 Gursoy et al. [39]	0.658	0.044	0.002	0.569	0.742
Classification 11 Gursoy et al. [39]	0.659	0.040	0.001	0.578	0.736
Classification 12 Gursoy et al. [39]	0.656	0.040	0.002	0.578	0.735
Classification 13 Gursoy et al. [39]	0.658	0.042	0.002	0.573	0.739
Classification 14 Izadi et al. [34]	0.643	0.058	0.002	0.529	0.755
Classification 15 Izadi et al. [34]	0.643	0.061	0.002	0.519	0.760

Classification 16 Izadi et al. [34]	0.641	0.056	0.001	0.528	0.749
BETWEEN-CLASSIFICATIONS SENSITIVITY AND SPECIFICITY (MMP8)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Sensitivity	0.725	0.096	0.001	0.546	0.919
Specificity	0.705	0.103	0.002	0.507	0.912

Appendix S3.2

SAMPLE SIZE (MMP9)					
	Total	**+ +**	**+ -**	**- +**	**- -**
Classification 1 Ramseier et al. [40]	79	27	12	12	28
Classification 2 Wu et al. [36]	57	26	14	4	13
Classification 3 Gursoy et al. [39]	117	33	14	3	67
Classification 4 Gursoy et al. [39]	110	27	14	2	67
Classification 5 Gursoy et al. [39]	165	28	14	56	67
Classification 6 Gursoy et al. [39]	230	88	14	61	67
WITHIN-CLASSIFICATION SENSITIVITY (MMP9)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Classification 1 Ramseier et al. [40]	0.701	0.070	0.001	0.558	0.829
Classification 2 Wu et al. [36]	0.863	0.061	0.001	0.736	0.965
Classification 3 Gursoy et al. [39]	0.898	0.049	0.001	0.794	0.977
Classification 4 Gursoy et al. [39]	0.906	0.053	0.001	0.794	0.986
Classification 5 Gursoy et al. [39]	0.347	0.051	0.001	0.251	0.451
Classification 6 Gursoy et al. [39]	0.593	0.039	0.001	0.515	0.669
WITHIN-CLASSIFICATION SPECIFICITY (MMP9)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Classification 1 Ramseier et al. [40]	0.714	0.066	0.002	0.581	0.838
Classification 2 Wu et al. [36]	0.544	0.089	0.002	0.365	0.713
Classification 3 Gursoy et al. [39]	0.816	0.041	0.000	0.733	0.893
Classification 4 Gursoy et al. [39]	0.817	0.041	0.000	0.733	0.892
Classification 5 Gursoy et al. [39]	0.831	0.039	0.001	0.749	0.903
Classification 6 Gursoy et al. [39]	0.826	0.038	0.002	0.745	0.895
BETWEEN-CLASSIFICATIONS SENSITIVITY AND SPECIFICITY (MMP9)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Sensitivity	0.703	0.233	0.002	0.212	1
Specificity	0.815	0.214	0.004	0.308	1

Appendix S3.3

SAMPLE SIZE (IL1beta)					
	Total	+ +	+ -	- +	- -
Classification 1 Afacan et al. [41]	60	32	2	8	18
Classification 2 Ramseier et al. [40]	79	21	18	18	22
Classification 3 Ebersole et al. [37]	80	44	2	6	28
Classification 4 Sanchez et al. [42]	74.5	46	0.5	13	15
Classification 5 Wu et al. [36]	57	25	13	5	14
Classification 6 Ebersole et al. [38]	209	76	26	25	82
WITHIN-CLASSIFICATION SENSITIVITY (IL1beta)					
	Estimate	Standard Dev.	MC Error	CI lower	CI upper
Classification 1 Afacan et al. [41]	0.804	0.055	0.000	0.685	0.901
Classification 2 Ramseier et al. [40]	0.572	0.074	0.003	0.423	0.711
Classification 3 Ebersole et al. [37]	0.870	0.044	0.000	0.780	0.948
Classification 4 Sanchez et al. [42]	0.794	0.050	0.001	0.686	0.881
Classification 5 Wu et al. [36]	0.784	0.077	0.003	0.627	0.918
Classification 6 Ebersole et al. [38]	0.750	0.040	0.001	0.667	0.825
WITHIN-CLASSIFICATION SPECIFICITY (IL1beta)					
	Estimate	Standard Dev.	MC Error	CI lower	CI upper
Classification 1 Afacan et al. [41]	0.873	0.064	0.001	0.740	0.976
Classification 2 Ramseier et al. [40]	0.569	0.074	0.002	0.427	0.717
Classification 3 Ebersole et al. [37]	0.914	0.047	0.001	0.814	0.988
Classification 4 Sanchez et al. [42]	0.923	0.063	0.003	0.792	1
Classification 5 Wu et al. [36]	0.596	0.093	0.003	0.402	0.762
Classification 6 Ebersole et al. [38]	0.763	0.039	0.001	0.685	0.837
BETWEEN-CLASSIFICATIONS SENSITIVITY AND SPECIFICITY (IL1beta)					
	Estimate	Standard Dev.	MC Error	CI lower	CI upper
Sensitivity	0.787	0.199	0.004	0.343	1
Specificity	0.780	0.199	0.003	0.346	1

Appendix S3.4

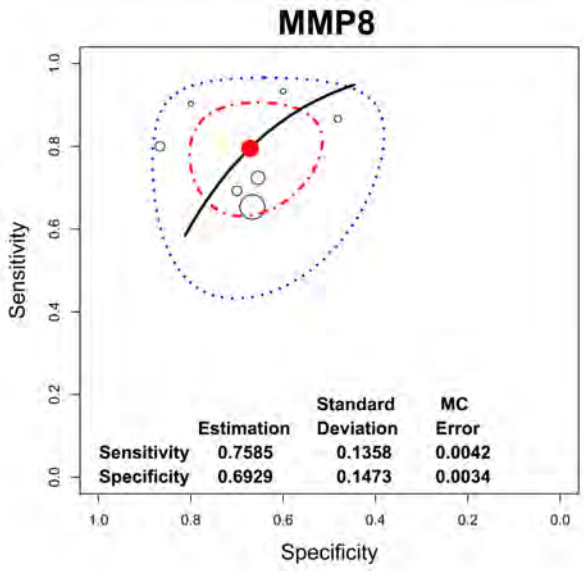
SAMPLE SIZE (IL6)					
	Total	**+ +**	**+ -**	**- +**	**- -**
Classification 1 Ramseier et al. [40]	79	23	16	16	24
Classification 2 Ebersole et al. [37]	80	44	1	6	29
Classification 3 Wu et al. [36]	57	16	14	14	13
Classification 4 Ebersole et al. [38]	209	79	23	22	85
WITHIN-CLASSIFICATION SENSITIVITY (IL6)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Classification 1 Ramseier et al. [40]	0.601	0.068	0.002	0.458	0.727
Classification 2 Ebersole et al. [37]	0.884	0.041	0.000	0.796	0.953
Classification 3 Wu et al. [36]	0.537	0.078	0.002	0.382	0.686
Classification 4 Ebersole et al. [38]	0.774	0.039	0.001	0.698	0.852
WITHIN-CLASSIFICATION SPECIFICITY (IL6)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Classification 1 Ramseier et al. [40]	0.610	0.069	0.002	0.473	0.744
Classification 2 Ebersole et al. [37]	0.927	0.045	0.001	0.832	0.994
Classification 3 Wu et al. [36]	0.521	0.083	0.003	0.354	0.679
Classification 4 Ebersole et al. [38]	0.789	0.037	0.001	0.713	0.858
BETWEEN-CLASSIFICATIONS SENSITIVITY AND SPECIFICITY (IL6)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Sensitivity	0.720	0.232	0.003	0.217	1
Specificity	0.731	0.230	0.002	0.227	1

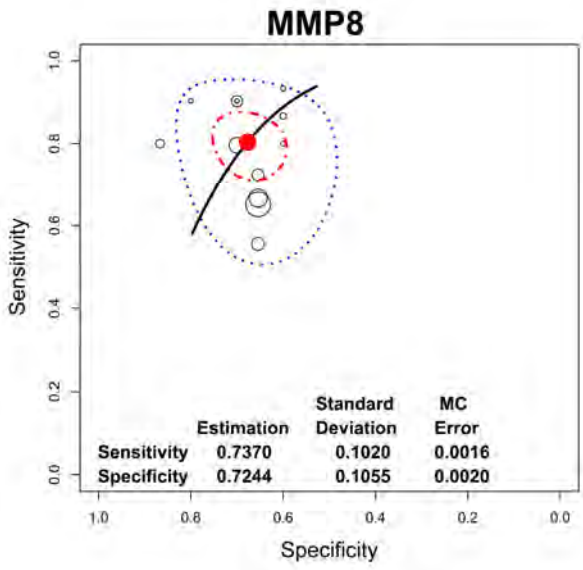
Appendix S3.5

SAMPLE SIZE (Haemoglobin)					
	Total	**+ +**	**+ -**	**- +**	**- -**
Classification 1 Pham et al. [44]	243	91	31	30	91
Classification 2 Nomura et al. [43]	92	41	9	13	29
Classification 3 Nomura et al. [45]	53	18	6	7	22
Classification 4 Nomura et al. [45]	37	6	11	3	17
WITHIN-CLASSIFICATION SENSITIVITY (Haemoglobin)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Classification 1 Pham et al. [44]	0.747	0.035	0.001	0.678	0.816
Classification 2 Nomura et al. [43]	0.750	0.046	0.001	0.654	0.837
Classification 3 Nomura et al. [45]	0.737	0.061	0.001	0.600	0.844
Classification 4 Nomura et al. [45]	0.728	0.092	0.002	0.524	0.893
WITHIN-CLASSIFICATION SPECIFICITY (Haemoglobin)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Classification 1 Pham et al. [44]	0.743	0.034	0.001	0.674	0.808
Classification 2 Nomura et al. [43]	0.747	0.048	0.001	0.649	0.839
Classification 3 Nomura et al. [45]	0.749	0.054	0.001	0.641	0.859
Classification 4 Nomura et al. [45]	0.692	0.070	0.002	0.537	0.802
BETWEEN-CLASSIFICATIONS SENSITIVITY AND SPECIFICITY (Haemoglobin)					
	Estimate	**Standard Dev.**	**MC Error**	**CI lower**	**CI upper**
Sensitivity	0.720	0.136	0.005	0.474	1
Specificity	0.752	0.132	0.005	0.513	1

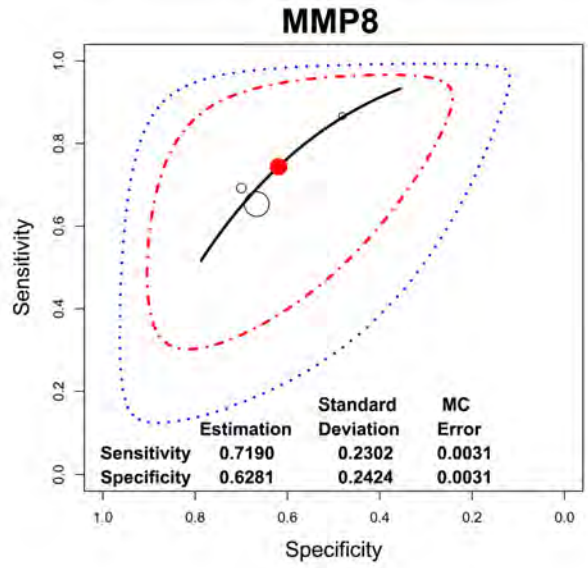
APPENDIX S4

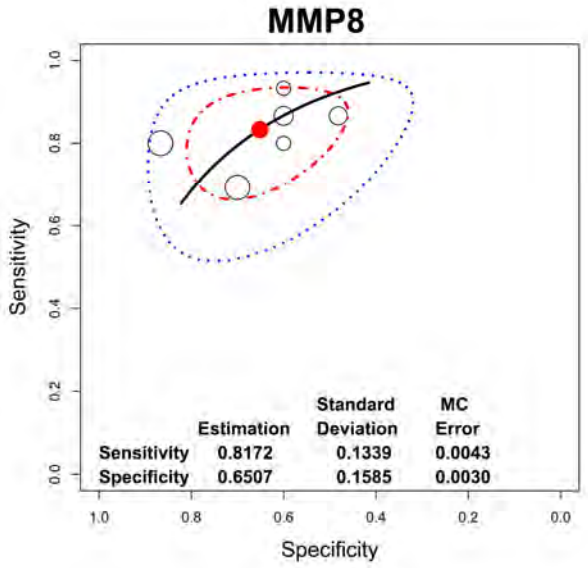
Appendix S4.1



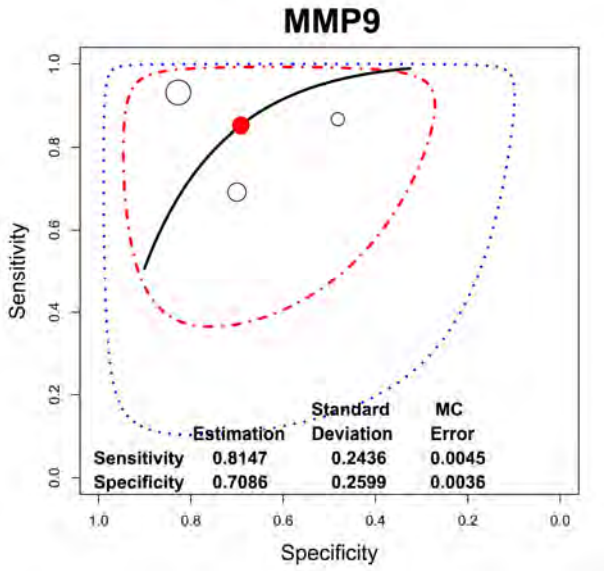


Appendix S4.2



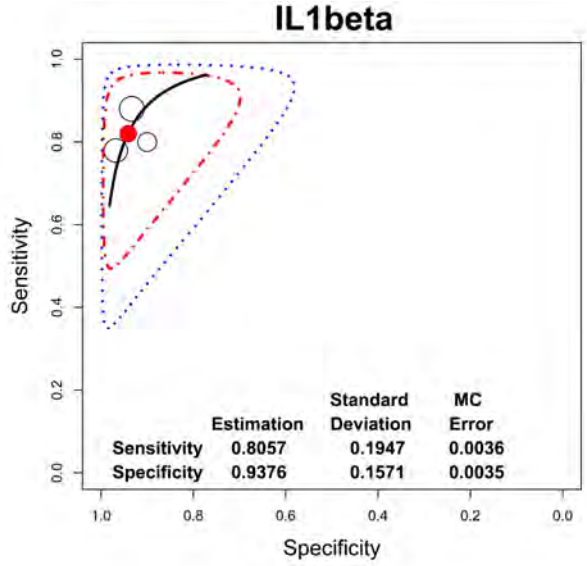


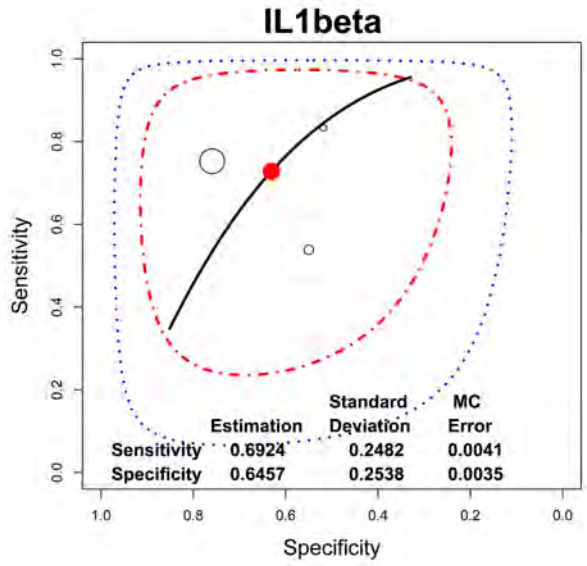
Appendix S5



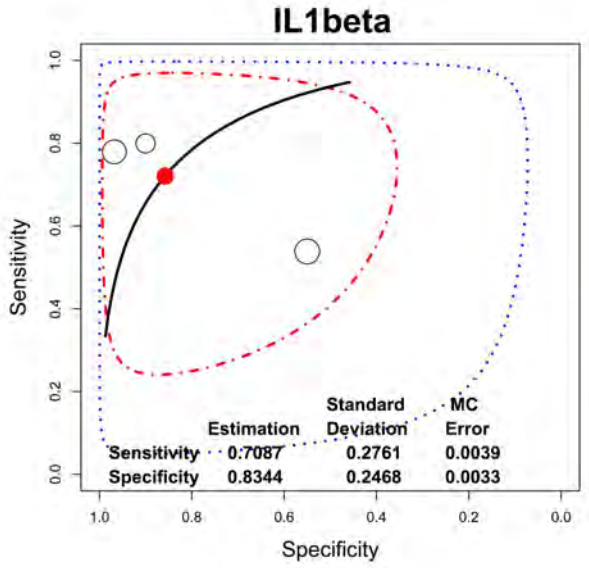
APPENDIX S6

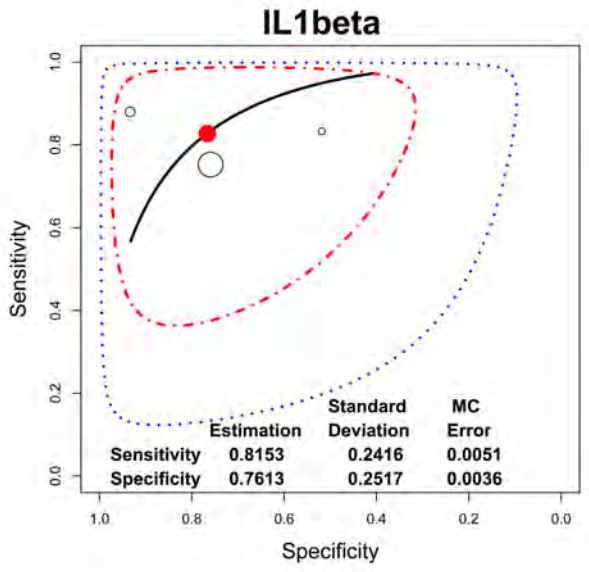
Appendix S6.1



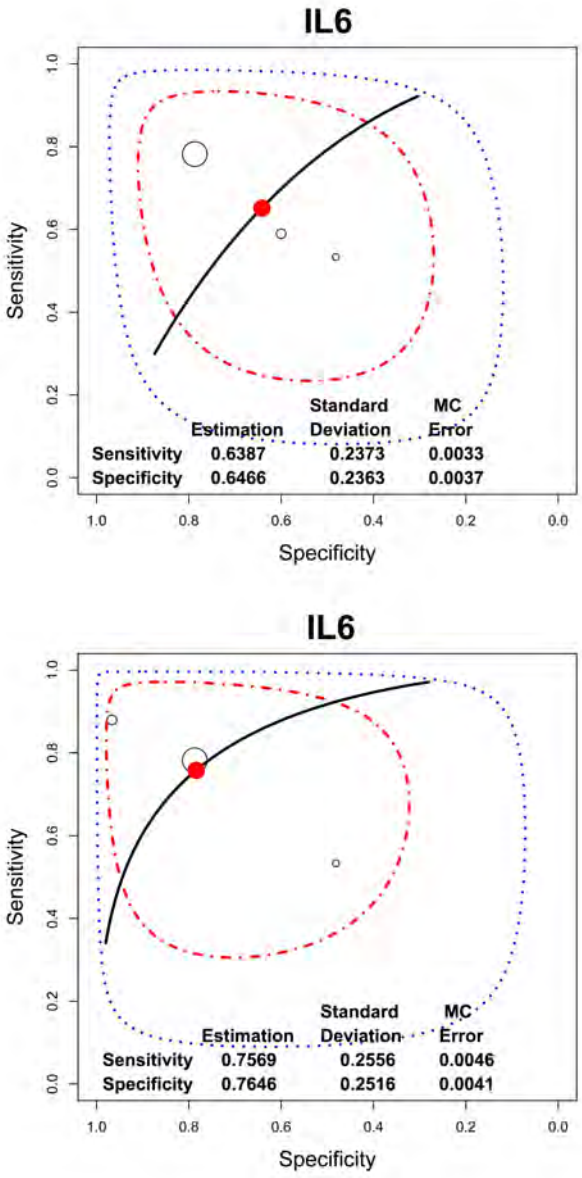


Appendix S6.2





APPENDIX S7


IL6
Sensitivity
Specificity
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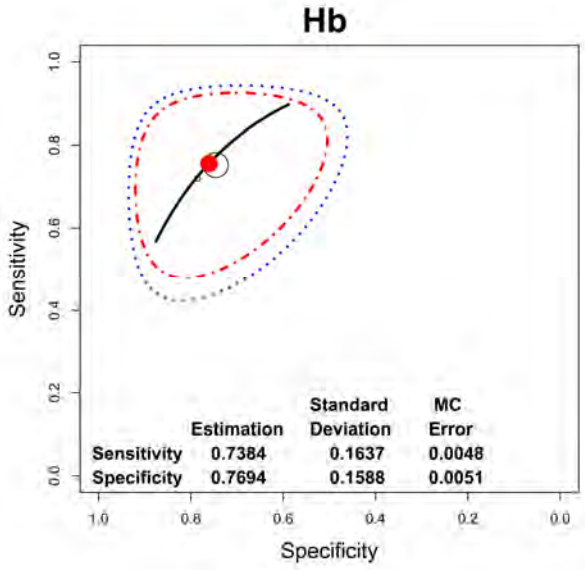
0.8
0.6
0.4
0.2
0.0
Estimation
Standard Deviation
MC Error
Sensitivity 0.6387 0.2373 0.0033
Specificity 0.6466 0.2363 0.0037
IL6
Sensitivity
Specificity
Estimation
Standard Deviation
MC Error
Sensitivity 0.7569 0.2556 0.0046
Specificity 0.7646 0.2516 0.0041

APPENDIX S8


Hb
Sensitivity
Specificity
1.0
0.8
0.6
0.4
0.2
0.0
Estimation
Standard Deviation
MC Error
Sensitivity 0.7384 0.1637 0.0048
Specificity 0.7694 0.1588 0.0051

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DE COMPOSTELA

Appendices Objective 3

Cytokine-based Predictive Models to Estimate the Probability of Chronic Periodontitis: Development of Diagnostic Nomograms.

Titles of the Appendices

Appendix S1. Approval of the study's protocol by the Clinical Research Ethics Committee of Galicia (number 2015/006).

Appendix S2. TRIPOD checklist.

Appendix S3. Boxplots for each cytokine both in the control group and in the perio group.

Appendix S4. Matrix Spearman correlations of cytokines.

Appendix S5. Additional information about the six cytokine-based models: confidence intervals of the model parameters.

Appendix S6. Additional information about the performance measures: confidence intervals of the discrimination and classification parameters.

APPENDIX S1

XUNTA DE GALICIA
CONSELLERÍA DE SANIDADE
Secretaría Xeral Técnica

Secretaría Técnica
Comité Autonómico de Ética da Investigación de Galicia
Secretaría Xeral. Consellería de Sanidade
Edificio Administrativo San Lázaro
15703 SANTIAGO DE COMPOSTELA
Tel. 881 546425; ceic@sergas.es

galicia

DITAME DO COMITÉ DE ÉTICA DA INVESTIGACIÓN DE SANTIAGO-LUGO

Juan Manuel Vázquez Lago, Secretario do Comité de Ética da Investigación de Santiago-Lugo

CERTIFICA:

Que este Comité avaliou na súa reunión do día 19/05/2015 o estudo:

Título: Asociación de la periodontitis crónica con las enfermedades sistémicas: análisis de la interacción microbioma-hospedador a nivel oral y sistémico
Promotor: Xunta de Galicia
Tipo de estudo: Outros
Versión:
Código do Promotor: EM 2014/025
Código de Rexistro: 2015/006

E, tomando en consideración as seguintes cuestións:

- A pertinencia do estudo, tendo en conta o coñecemento dispoñible, así coma os requisitos legais aplicables, e en particular a Lei 14/2007, de investigación biomédica, o Real Decreto 1716/2011, de 18 de novembro, polo que se establecen os requisitos básicos de autorización e funcionamento dos biobancos con fins de investigación biomédica e do tratamento das mostras biolóxicas de orixe humana, e se regula o funcionamento e organización do Rexistro Nacional de Biobancos para investigación biomédica, a ORDE SAS/3470/2009, de 16 de decembro, pola que se publican as Directrices sobre estudos Posautorización de Tipo Observacional para medicamentos de uso humano, e a Circular nº 07/2004, investigacións clínicas con produtos sanitarios.
- A idoneidade do protocolo en relación cos obxectivos do estudo, xustificación dos riscos e molestias previsibles para o suxeito, así coma os beneficios esperados.
- Os principios éticos da Declaración de Helsinki vixente.
- Os Procedementos Normalizados de Traballo do Comité.

Emite un **INFORME FAVORABLE** para a realización do estudo **polo/a investigador/a do centro:**

Centros	Investigadores Principais
USC; Facultade de Medicina e Odontoloxía	Inmaculada Tomás Carmona

En Santiago de Compostela, a 25 de maio de 2015
O secretario NOMBRE VAZQUEZ LAGO JUAN MANUEL - NIF 44829259M

Juan M. Vázquez Lago

APPENDIX S2

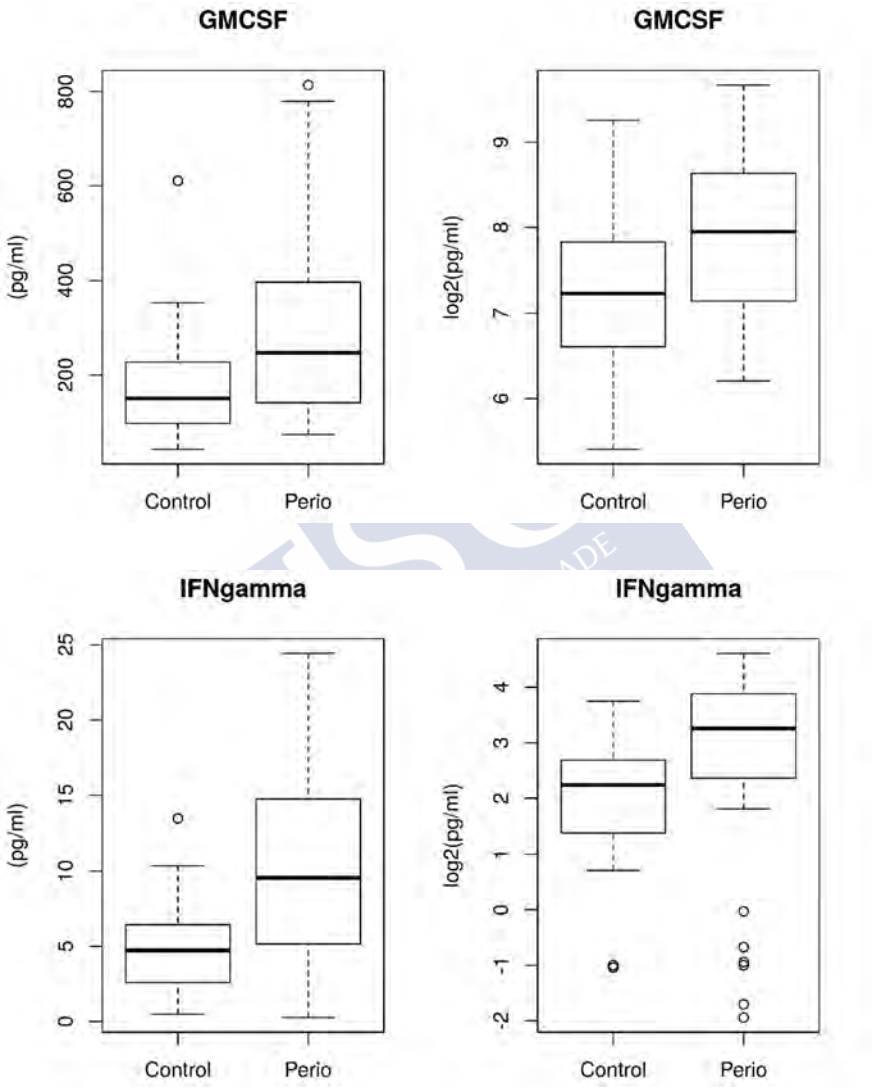
Section/Topic	Item		Checklist Item (TRIPOD Checklist)	Reported on Page
TITLE AND ABSTRACT				
Title	1	D;V	Identify the study as developing and/or validating a multivariable prediction model, the target population, and the outcome to be predicted	319
Abstract	2	D;V	Provide a summary of objectives, study design, setting, participants, sample size, predictors, outcome, statistical analysis, results, and conclusions	319, 320
INTRODUCTION				
Background and objectives	3a	D;V	Explain the medical context (including whether diagnostic or prognostic) and rationale for developing or validating the multivariable prediction model, including references to existing models	321-323
	3b	D;V	Specify the objectives, including whether the study describes the development or validation of the model or both	323
METHODS				
Source of data	4a	D;V	Describe the study design or source of data (e.g., randomized trial, cohort, or registry data), separately for the development and validation data sets, if applicable	323
	4b	D;V	Specify the key study dates, including start of accrual; end of accrual; and, if applicable, end of follow-up	323
Participants	5a	D;V	Specify key elements of the study setting (e.g., primary care, secondary care, general population) including number and location of centres	323, Figure 1
	5b	D;V	Describe eligibility criteria for participants	323, 324
	5c	D;V	Give details of treatments received, if relevant	N/A
Outcome	6a	D;V	Clearly define the outcome that is predicted by the prediction model, including how and when assessed	324, 325
	6b	D;V	Report any actions to blind assessment of the outcome to be predicted	324, 325
Predictors	7a	D;V	Clearly define all predictors used in developing the multivariable prediction model, including how and when they were measured	325-328 Figure 2
	7b	D;V	Report any actions to blind assessment of predictors for the outcome and other predictors	326
Sample size	8	D;V	Explain how the study size was arrived at	329
Missing data	9	D;V	Describe how missing data were handled (e.g., complete-case analysis, single	328

			imputation, multiple imputation) with details of any imputation method	
Statistical analysis methods	10a	D	Describe how predictors were handled in the analyses	329,330, Figure 2
	10b	D	Specify type of model, all model-building procedures (including any predictor selection), and method for internal validation	329, 330, Figure 2
	10c	V	For validation, describe how the predictions were calculated	N/A
	10d	D;V	Specify all measures used to assess model performance and, if relevant, to compare multiple models	330 Figure 2
	10e	V	Describe any model updating (e.g., recalibration) arising from the validation, if done	N/A
Risk groups	11	D;V	Provide details on how risk groups were created, if done	N/A
Development vs. validation	12	V	For validation, identify any differences from the development data in setting, eligibility criteria, outcome, and predictors	N/A
RESULTS				
Participants	13a	D;V	Describe the flow of participants through the study, including the number of participants with and without the outcome and, if applicable, a summary of the follow-up time. A diagram may be helpful	324, Figure 1
	13b	D;V	Describe the characteristics of the participants (basic demographics, clinical features, available predictors), including the number of participants with missing data for predictors and outcome	332, 333, Table 1
	13c	V	For validation, show a comparison with the development data of the distribution of important variables (demographics, predictors and outcome)	N/A
Model development	14a	D	Specify the number of participants and outcome events in each analysis	335
	14b	D	If done, report the unadjusted association between each candidate predictor and outcome	334, 335 Tables 2 Appendix S3 Appendix S4
Model specification	15a	D	Present the full prediction model to allow predictions for individuals (i.e., all regression coefficients, and model intercept or baseline survival at a given time point)	335, 336, Table 3 Appendix S5
	15b	D	Explain how to use the prediction model	344, Figures 5, Figures 6

Model performance	16	D;V	Report performance measures (with CIs) for the prediction model	336, 337, Table 4, Figures 3, Figures 4 Appendix S6
Model-updating	17	V	If done, report the results from any model updating (i.e., model specification, model performance)	N/A
DISCUSSION				
Limitations	18	D;V	Discuss any limitations of the study (such as nonrepresentative sample, few events per predictor, missing data)	358
Interpretation	19a	V	For validation, discuss the results with reference to performance in the development data, and any other validation data	N/A
	19b	D;V	Give an overall interpretation of the results, considering objectives, limitations, results from similar studies, and other relevant evidence	351-358
Implications	20	D;V	Discuss the potential clinical use of the model and implications for future research	358, 359
OTHER INFORMATION				
Supplementary information	21	D;V	Provide information about the availability of supplementary resources, such as study protocol, Web calculator, and data sets	325, Appendix S1
Funding	22	D;V	Give the source of funding and the role of the funders for the present study	320

*Items relevant only to the development of a prediction model are denoted by D, items relating solely to a validation of a prediction model are denoted by V, and items relating to both are denoted D;V.

APPENDIX S3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Perio
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9
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IFNgamma
(pg/ml)
0
5
10
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IFNgamma
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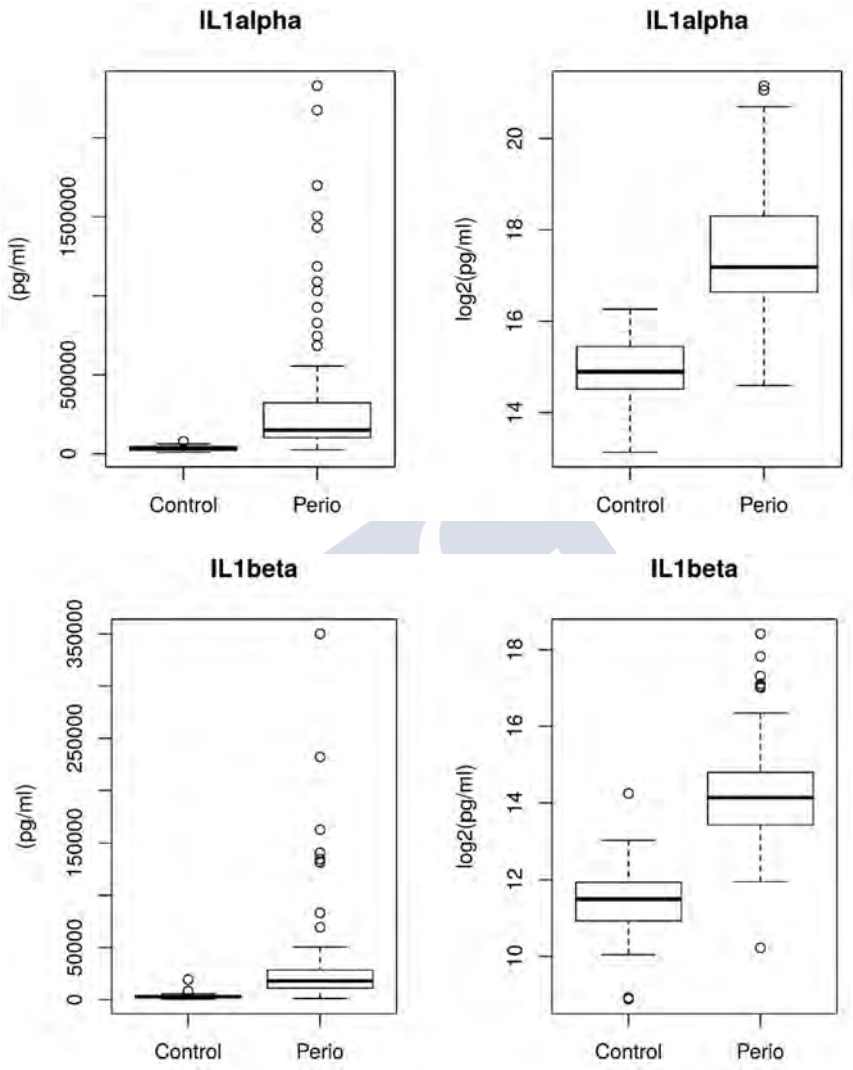
1
2
3
4
Control
Perio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(pg/ml)
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250000
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10
12
14
16
18
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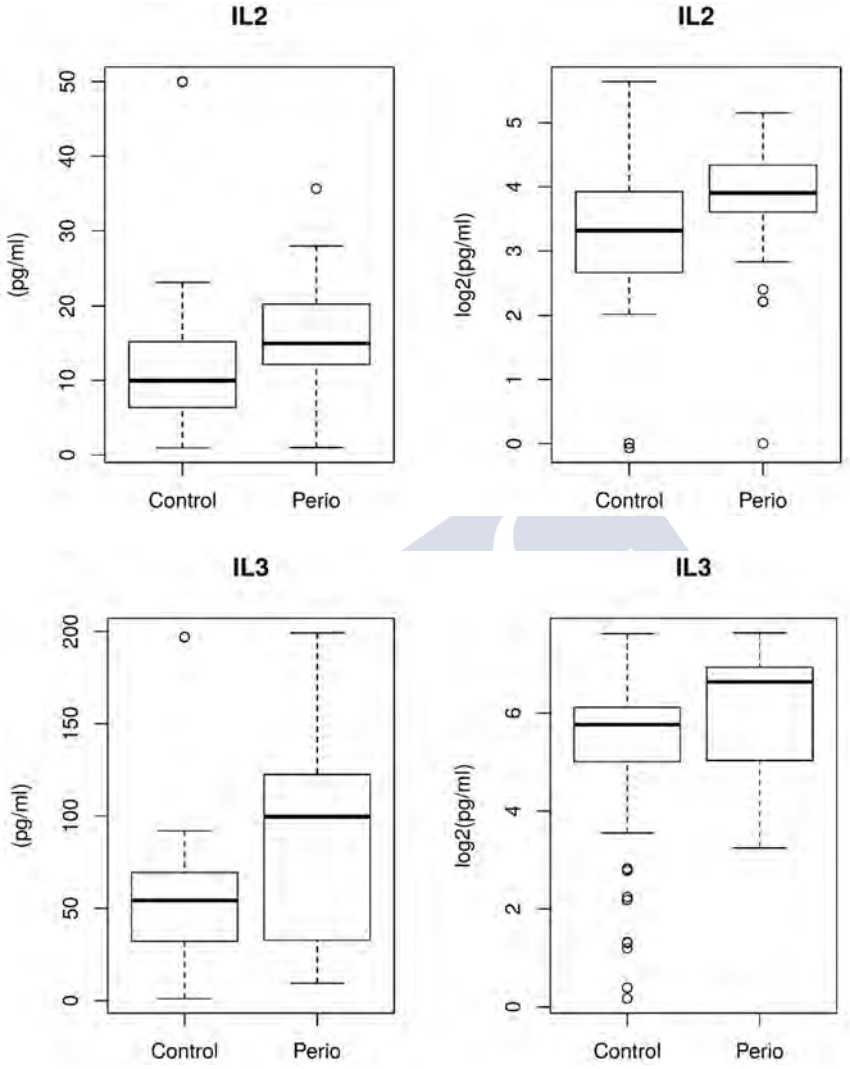
Perio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Perio
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(pg/ml)
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200
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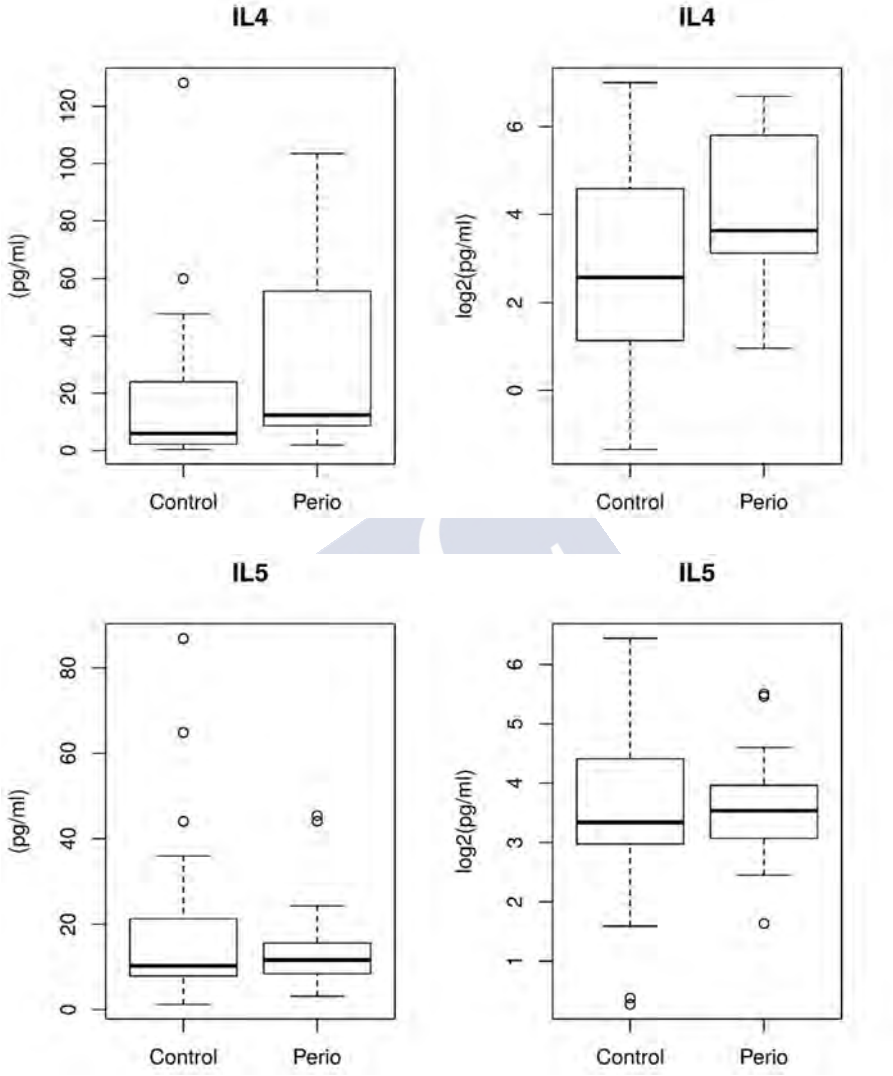
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(pg/ml)
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5
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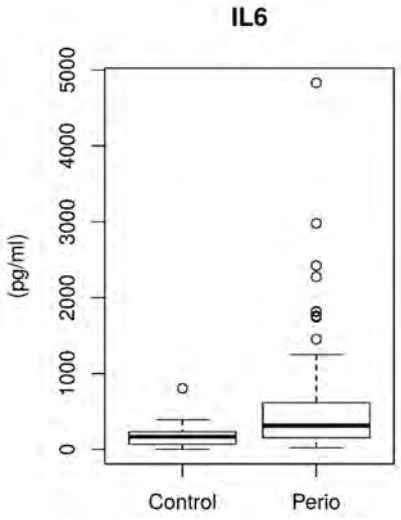
Perio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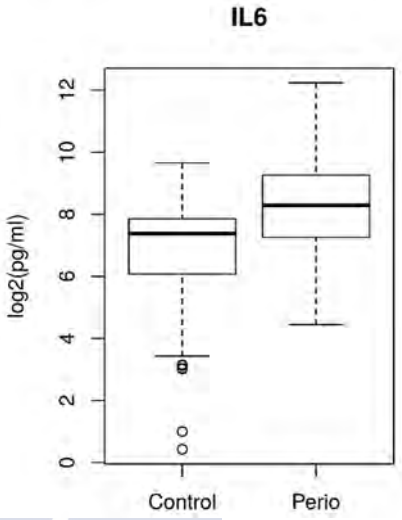
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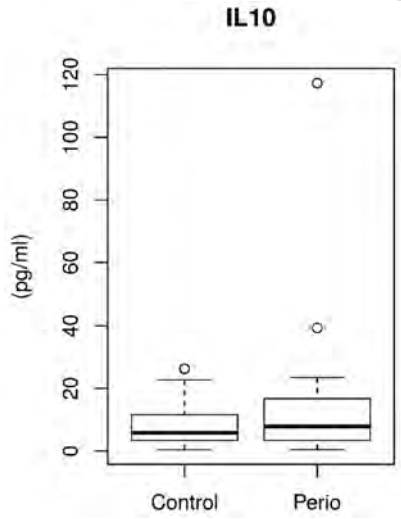
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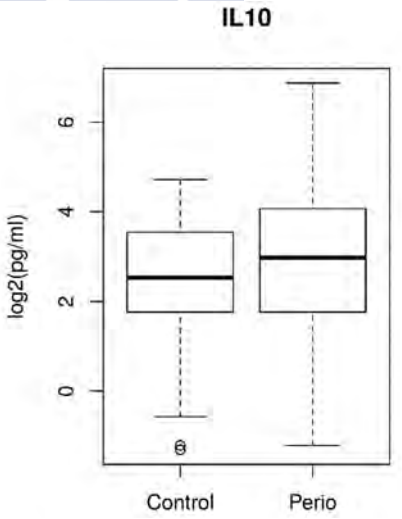
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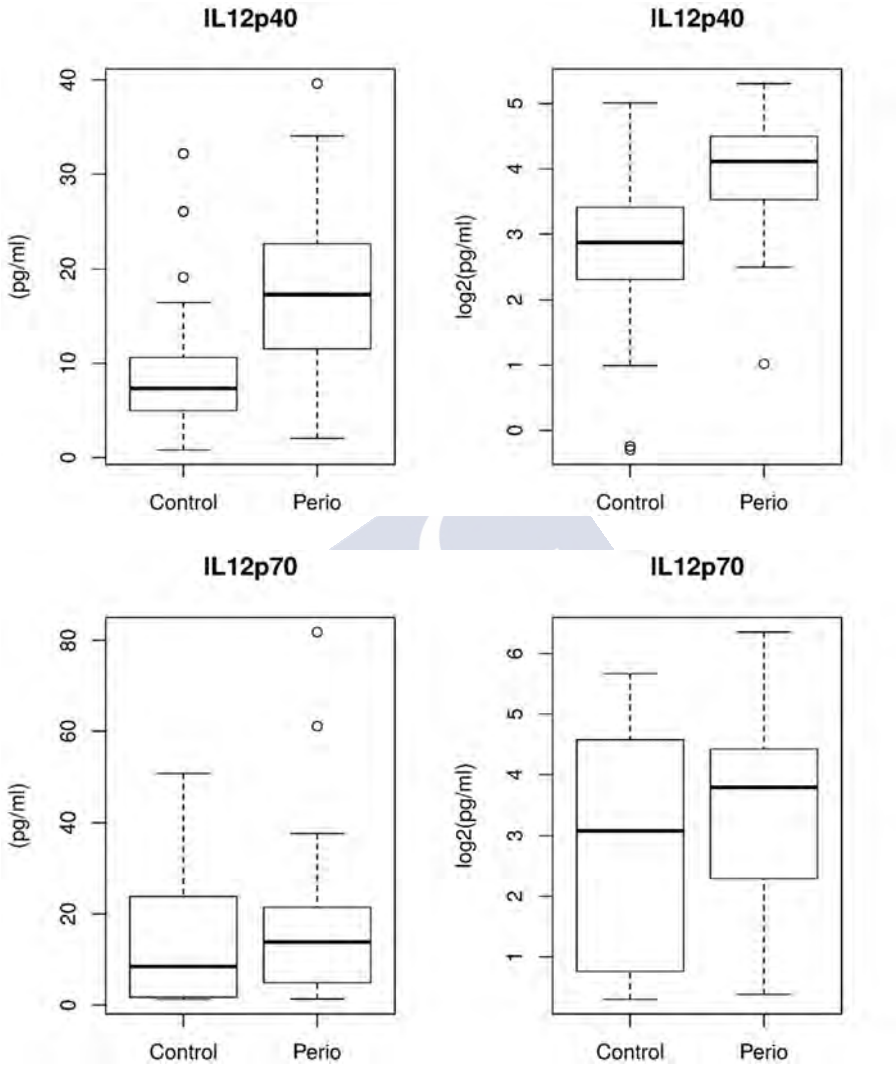
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(pg/ml)
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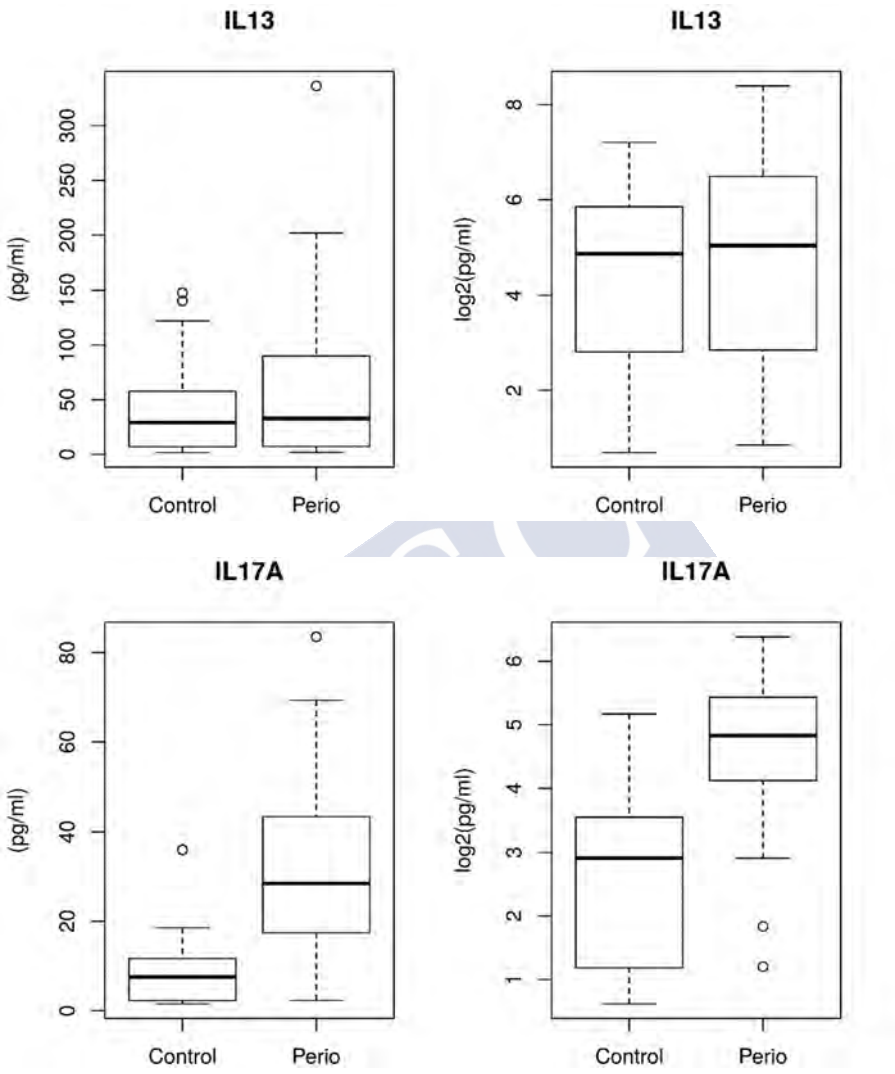
3
4
5
6
Control
Perio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300
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Perio
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4
6
8
Control
Perio
IL17A
(pg/ml)
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40
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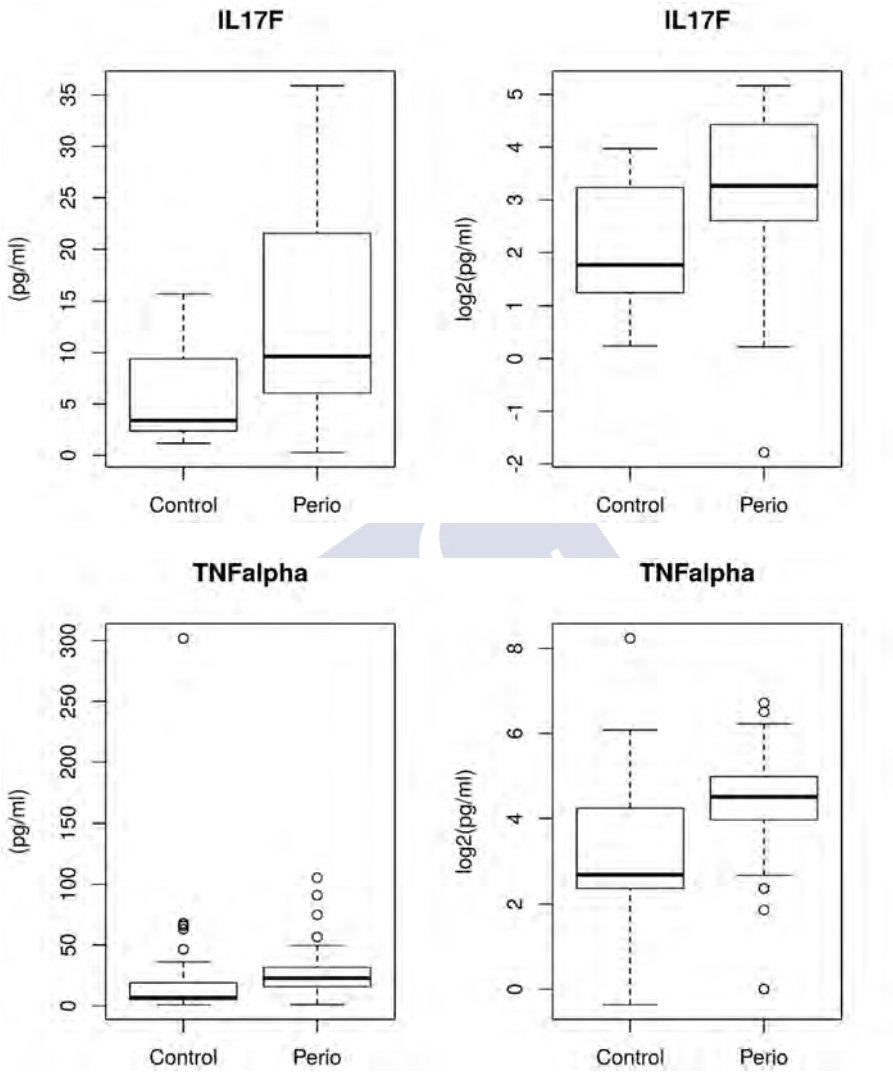
Perio
IL17A
log2(pg/ml)
1
2
3
4
5
6
Control
Perio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-1
0
1
2
3
4
5
Control
Perio
TNFalpha
(pg/ml)
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100
150
200
250
300
Control
Perio
TNFalpha
log2(pg/ml)
0
2
4
6
8
Control
Perio

APPENDIX S4

	GMCSF	IL1 alpha	IL1 beta	IL6	IL12 p40	IL17A	IL17F	TNF alpha	IFN gamma	IL2	IL3	IL4	IL5	IL10	IL12 p70	IL13
GMCSF	1	0.32	0.25	0.34	0.46	0.22	0.45	0.33	-0.00	0.27	-0.00	0.36	0.30	-0.20	0.11	-0.34
IL1alpha	0.32	1	0.95	0.49	0.61	0.87	0.56	0.41	0.64	0.48	0.51	0.40	0.14	0.35	0.21	0.24
IL1beta	0.25	0.95	1	0.46	0.62	0.88	0.57	0.42	0.64	0.43	0.48	0.35	0.05	0.34	0.17	0.25
IL6	034	0.49	0.46	1	0.35	0.52	0.35	0.37	0.40	0.31	0.38	0.33	0.35	0.33	0.34	0.28
IL12p40	0.46	0.61	0.62	0.35	1	0.53	0.73	0.32	0.33	0.43	0.26	0.58	0.08	-0.09	0.21	-0.11
IL17A	0.22	0.87	0.88	0.52	0.53	1	0.60	0.58	0.80	0.56	0.70	0.49	0.28	0.56	0.40	0.46
IL17F	0.45	0.56	0.57	0.35	0.73	0.60	1	0.61	0.51	0.50	0.42	0.79	0.17	0.06	0.45	-0.03
TNFalpha	0.33	0.41	0.42	0.37	0.32	0.58	0.61	1	0.60	0.49	0.55	0.69	0.38	0.45	0.65	0.26
IFNgamma	-0.00	0.64	0.64	0.40	0.33	0.80	0.51	0.60	1	0.63	0.88	0.61	0.32	0.65	0.64	0.69
IL2	0.27	0.48	0.43	0.31	0.43	0.56	0.50	0.49	0.63	1	0.67	0.64	0.39	0.42	0.55	0.42
IL3	-0.00	0.51	0.48	0.38	0.26	0.70	0.42	0.55	0.88	0.67	1	0.63	0.35	0.59	0.71	0.69
IL4	0.36	0.40	0.35	0.33	0.58	0.49	0.79	0.69	0.61	0.64	0.63	1	0.40	0.20	0.78	0.19
IL5	0.30	0.14	0.05	0.35	0.08	0.28	0.17	0.38	0.32	0.39	0.35	0.40	1	0.35	0.56	0.35
IL10	-0.20	0.35	0.34	0.33	-0.09	0.56	0.06	0.45	0.65	0.42	0.59	0.20	0.35	1	0.49	0.78
IL12p70	0.11	0.21	0.17	0.34	0.21	0.40	0.45	0.65	0.64	0.55	0.71	0.78	0.56	0.49	1	0.49
IL13	-0.34	0.24	0.25	0.28	-0.11	0.46	-0.03	0.26	0.69	0.42	0.69	0.19	0.35	0.78	0.49	1

APPENDIX S5

One-Cytokine Models	Intercept 95% CIs	Variable Cytokine 95% CIs	Variable Smoking Current 95% CIs
-60.732 + 3.133 **IL1alpha** + 1.783 **SmokingCurrent**	-82.979 -38.486	1.970 4.297	0.384 3.183
-34.825 + 2.136 **IL1beta** + 1.722 **SmokingCurrent**	-45.979 -23.672	1.440 2.831	0.433 3.010
-13.663 + 1.823 **IL17A** + 1.860 **SmokingCurrent**	-18.233 -9.093	1.200 2.445	0.742 2.978

Two-Cytokine Models	Intercept 95% CIs	Variable Pro-Cytokine 95% CIs	Variable Anti-Cytokine 95% CIs	Variable Current Smoking 95% CIs
-82.932 + 4.622 **IL1alpha** -1.146 **IFNgamma** + 2.042 **SmokingCurrent**	-117.209 -48.654	2.674 6.570	-1.899 -0.392	0.441 3.644
-34.875 + 2.331 **IL1beta** -0.505 **IL10** + 1.701 **SmokingCurrent**	-46.299 -23.452	1.556 3.106	-0.985 -0.026	0.336 3.066
-18.546 + 5.024 **IL17A** - 3.167 **IFNgamma** + 2.984 **SmokingCurrent**	-24.962 -12.130	3.035 7.014	-4.872 -1.463	1.257 4.712

APPENDIX S6

Smoking adjusted Model	AUC 95% CIs	ACC (%) 95% CIs	SENS (%) 95% CIs	SPEC (%) 95% CIs	PPV (%) 95% CIs	NPV (%) 95% CIs
IL1alpha	0.948 0.997	89.1 97.2	89.0 98.6	85.1 97.2	86.0 97.2	89.4 98.6
IL1beta	0.963 0.930	89.7 97.2	87.6 97.2	89.1 98.6	89.1 98.6	87.9 97.3
IL17A	0.937 0.898	83.6 93.8	80.8 95.8	81.0 95.9	82.4 95.5	82.9 95.5

Smoking adjusted Model	AUC 95% CIs	ACC (%) 95% CIs	SENS (%) 95% CIs	SPEC (%) 95% CIs	PPV (%) 95% CIs	NPV (%) 95% CIs
IL1alpha + IFNgamma	0.971 0.999	91.1 98.6	86.3 98.6	93.2 100.0	93.0 100.0	87.8 98.6
IL1beta + IL10	0.945 0.997	90.4 97.9	89.0 98.6	89.1 98.6	89.3 98.6	89.6 98.6
IL17A + IFNgamma	0.950 0.997	87.7 96.5	83.5 95.8	89.1 98.6	88.7 98.5	85.1 96.0

Appendices Objective 4

Cytokine Thresholds in Gingival Crevicular Fluid with Potential Diagnosis of Chronic Periodontitis Differentiating by Smoking Status.

Titles of the Appendices

Appendix S1. Approval of the study's protocol by the Clinical Research Ethics Committee of Galicia (number 2015/006).

Appendix S2. TRIPOD checklist.

Appendix S3. Additional information about the six cytokine-based models: confidence intervals of the model parameters.

Appendix S4. Additional information about the performance measures: confidence intervals of the discrimination and classification parameters.

APPENDIX S1

XUNTA DE GALICIA
CONSELLERÍA DE SANIDADE
Secretaría Xeral Técnica

Secretaría Técnica
Comité Autonómico de Ética da Investigación de Galicia
Secretaría Xeral. Consellería de Sanidade
Edificio Administrativo San Lázaro
15703 SANTIAGO DE COMPOSTELA
Tel: 881 546425 ceic@sergas.es

galicia

DITAME DO COMITÉ DE ÉTICA DA INVESTIGACIÓN DE SANTIAGO-LUGO

Juan Manuel Vázquez Lago, Secretario do Comité de Ética da Investigación de Santiago-Lugo

CERTIFICA:

Que este Comité avaliou na súa reunión do día 19/05/2015 o estudo:

Título: Asociación de la periodontitis crónica con las enfermedades sistémicas: análisis de la interacción microbioma-hospedador a nivel oral y sistémico
Promotor: Xunta de Galicia
Tipo de estudo: Outros
Versión:
Código do Promotor: EM 2014/025
Código de Rexistro: 2015/006

E, tomando en consideración as seguintes cuestións:

- A pertinencia do estudo, tendo en conta o coñecemento dispoñible, así coma os requisitos legais aplicables, e en particular a Lei 14/2007, de investigación biomédica, o Real Decreto 1716/2011, de 18 de novembro, polo que se establecen os requisitos básicos de autorización e funcionamento dos biobancos con fins de investigación biomédica e do tratamento das mostras biolóxicas de orixe humana, e se regula o funcionamento e organización do Rexistro Nacional de Biobancos para investigación biomédica, a ORDE SAS/3470/2009, de 16 de decembro, pola que se publican as Directrices sobre estudos Posautorización de Tipo Observacional para medicamentos de uso humano, e a Circular nº 07/2004, investigacións clínicas con produtos sanitarios.
- A idoneidade do protocolo en relación cos obxectivos do estudo, xustificación dos riscos e molestias previsibles para o suxeito, así coma os beneficios esperados.
- Os principios éticos da Declaración de Helsinki vixente.
- Os Procedementos Normalizados de Traballo do Comité.

Emite un **INFORME FAVORABLE** para a realización do estudo **polo/a investigador/a do centro:**

Centros	Investigadores Principais
USC; Facultade de Medicina e Odontoloxía	Inmaculada Tomás Carmona

En Santiago de Compostela, a 25 de maio de 2015
O secretario NOMBRE VAZQUEZ LAGO JUAN MANUEL - NIF 44829259M

Juan M. Vázquez Lago

APPENDIX S2

Section/Topic	Item		Checklist Item (TRIPOD Checklist)	Reported on Page
TITLE AND ABSTRACT				
Title	1	D;V	Identify the study as developing and/or validating a multivariable prediction model, the target population, and the outcome to be predicted	371
Abstract	2	D;V	Provide a summary of objectives, study design, setting, participants, sample size, predictors, outcome, statistical analysis, results, and conclusions	371, 372
INTRODUCTION				
Background and objectives	3a	D;V	Explain the medical context (including whether diagnostic or prognostic) and rationale for developing or validating the multivariable prediction model, including references to existing models	373, 374
	3b	D;V	Specify the objectives, including whether the study describes the development or validation of the model or both	374
METHODS				
Source of data	4a	D;V	Describe the study design or source of data (e.g., randomized trial, cohort, or registry data), separately for the development and validation data sets, if applicable	375
	4b	D;V	Specify the key study dates, including start of accrual; end of accrual; and, if applicable, end of follow-up	375
Participants	5a	D;V	Specify key elements of the study setting (e.g., primary care, secondary care, general population) including number and location of centres	375
	5b	D;V	Describe eligibility criteria for participants	375
	5c	D;V	Give details of treatments received, if relevant	N/A
Outcome	6a	D;V	Clearly define the outcome that is predicted by the prediction model, including how and when assessed	375
	6b	D;V	Report any actions to blind assessment of the outcome to be predicted	N/A
Predictors	7a	D;V	Clearly define all predictors used in developing the multivariable prediction model, including how and when they were measured	376, 377 Figure 1
	7b	D;V	Report any actions to blind assessment of predictors for the outcome and other predictors	377
Sample size	8	D;V	Explain how the study size was arrived at	379

Missing data	9	D;V	Describe how missing data were handled (e.g., complete-case analysis, single imputation, multiple imputation) with details of any imputation method	377
Statistical analysis methods	10a	D	Describe how predictors were handled in the analyses	379, 380 Figure 1
	10b	D	Specify type of model, all model-building procedures (including any predictor selection), and method for internal validation	380, Figure 1
	10c	V	For validation, describe how the predictions were calculated	N/A
	10d	D;V	Specify all measures used to assess model performance and, if relevant, to compare multiple models	380, Figure 1
	10e	V	Describe any model updating (e.g., recalibration) arising from the validation, if done	N/A
Risk groups	11	D;V	Provide details on how risk groups were created, if done	379
Development vs. validation	12	V	For validation, identify any differences from the development data in setting, eligibility criteria, outcome, and predictors	N/A
RESULTS				
Participants	13a	D;V	Describe the flow of participants through the study, including the number of participants with and without the outcome and, if applicable, a summary of the follow-up time. A diagram may be helpful	N/A
	13b	D;V	Describe the characteristics of the participants (basic demographics, clinical features, available predictors), including the number of participants with missing data for predictors and outcome	381, Table 1
	13c	V	For validation, show a comparison with the development data of the distribution of important variables (demographics, predictors and outcome)	N/A
Model development	14a	D	Specify the number of participants and outcome events in each analysis	379
	14b	D	If done, report the unadjusted association between each candidate predictor and outcome	382, 383 Tables 2
Model specification	15a	D	Present the full prediction model to allow predictions for individuals (i.e., all regression coefficients, and model intercept or baseline survival at a given time point)	384,Figure 2, Figure 3, Figure 4, Appendix S3
	15b	D	Explain how to use the prediction model	384,Figure 2, Figure 3, Figure 4

Model performance	16	D;V	Report performance measures (with CIs) for the prediction model	384, 385, Tables 3, Appendix S4
Model-updating	17	V	If done, report the results from any model updating (i.e., model specification, model performance)	N/A
DISCUSSION				
Limitations	18	D;V	Discuss any limitations of the study (such as nonrepresentative sample, few events per predictor, missing data)	394
Interpretation	19a	V	For validation, discuss the results with reference to performance in the development data, and any other validation data	N/A
	19b	D;V	Give an overall interpretation of the results, considering objectives, limitations, results from similar studies, and other relevant evidence	389-394
Implications	20	D;V	Discuss the potential clinical use of the model and implications for future research	393, 394
OTHER INFORMATION				
Supplementary information	21	D;V	Provide information about the availability of supplementary resources, such as study protocol, Web calculator, and data sets	375, 376, Appendix S1
Funding	22	D;V	Give the source of funding and the role of the funders for the present study	372

*Items relevant only to the development of a prediction model are denoted by D, items relating solely to a validation of a prediction model are denoted by V, and items relating to both are denoted D;V.

APPENDIX S3

Cytokine Model (Smokers)	Intercept 95% CIs	Variable Cytokine 95% CIs
-4.521 + 9.237e-05 **IL1alpha**	-7.847 -1.195	2.7e-05 0.000
-3.601 + 0.000 **IL1beta**	-6.157 -1.044	0.000 0.001
-2.974 + 0.272 **IL17A**	-5.231 -0.717	0.107 0.437
Cytokine Ratio Model (Smokers)	**Intercept 95% CIs**	**Variable Ratio 95% CIs**
-2.274 + 0.000 **IL1alpha/IL2**	-4.311 -0.236	0.000 0.000
-1.355 + 0.003 **IL1beta/IL2**	-2.817 0.105	0.001 0.006
-4.331 + 5.343 **IL17A/IL2**	-7.366 -1.296	1.999 8.688

Cytokine Model (Non-Smokers)	Intercept 95% CIs	Variable Cytokine 95% CIs
-5.709 + 7.93e-05 **IL1alpha**	-8.108 -3.310	3.8e-05 0.000
-3.584 + 0.000 **IL1beta**	-4.842 -2.326	2e-04 0.000
-3.3645 + 0.161 **IL17A**	-4.569 -2.159	0.091 0.231
Cytokine Ratio Model (Non-Smokers)	**Intercept 95% CIs**	**Variable Ratio 95% CIs**
-2.025 + 0.000 **IL1alpha/IL2**	-2.832 -1.218	6.6e-05 0.000
-1.777 + 0.001 **IL1beta/IL2**	-2.501 -1.0537	0.000 0.001
-1.736 + 0.689 **IL17A/IL12**	-2.527 -0.945	0.264 1.113

APPENDIX S4

Models (Smokers)	AUC 95% CIs	ACC (%) 95% CIs	SENS (%) 95% CIs	SPEC (%) 95% CIs	PPV (%) 95% CIs	NPV (%) 95% CIs
IL1alpha	0.925 1.000	87.0 98.1	100.0 100.0	46.1 92.3	85.4 97.6	100.0 100.0
IL1beta	0.923 1.000	88.8 100.0	92.6 100.0	61.5 100.0	88.8 100.0	75.0 100.0
IL17A	0.872 1.000	85.1 98.1	87.8 100.0	61.5 100.0	88.8 100.0	66.6 100.0
IL1alpha/IL2	0.878 0.781	79.6 92.5	100.0 100.0	15.3 69.2	78.8 91.1	100.0 100.0
IL1beta/IL2	0.906 0.798	85.1 98.1	87.8 100.0	61.5 100.0	88.6 100.0	68.7 100.0
IL171/IL2	0.954 0.904	87.0 98.1	100.0 100.0	46.1 92.3	85.4 97.6	68.7 100.0

Models (Non-Smokers)	AUC 95% CIs	ACC (%) 95% CIs	SENS (%) 95% CIs	SPEC (%) 95% CIs	PPV (%) 95% CIs	NPV (%) 95% CIs
IL1alpha	0.910 1.000	88.1 97.8	75.0 96.8	91.8 100.0	84.3 100.0	88.0 98.3
IL1beta	0.880 1.000	89.2 98.9	78.1 100.0	91.8 100.0	84.8 100.0	89.5 100.0
IL17A	0.848 0.980	81.7 94.6	62.5 90.6	86.8 98.3	75.0 96.4	82.8 95.1
IL1alpha/IL2	0.854 0.968	81.6 94.6	71.8 96.8	81.9 96.7	70.5 93.5	85.2 98.0
IL1beta/IL2	0.816 0.955	77.4 91.3	65.6 93.7	77.0 95.0	64.7 89.6	82.8 96.3
IL17A/IL2	0.779 0.934	72.0 88.1	75.0 96.8	65.5 86.8	56.8 78.3	85.0 98.0

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Appendices Objective 5

Diagnostic Accuracy of IL1beta in Saliva: The Development of Predictive Models for Estimating the Probability of the Occurrence of Periodontitis in Non-Smokers and Smokers.

Titles of the Appendices

Appendix S1. Approval of the study's protocol by the Clinical Research Ethics Committee of Galicia (number 2015/006).

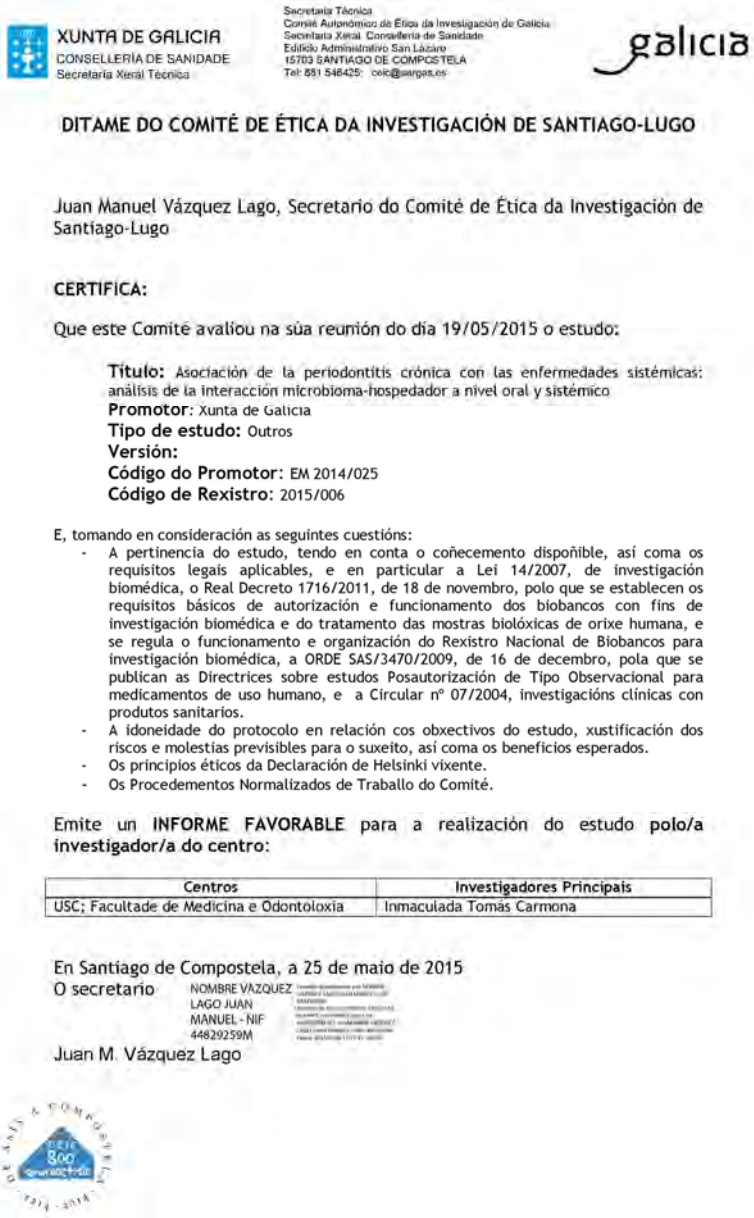
Appendix S2. TRIPOD checklist.

Appendix S3. Age, gender, smoking habit and clinical characteristics associated with periodontal status in the control and non-treated perio groups, as well as in the periodontal patients undergoing non-surgical periodontal treatment before and after treatment; In non-smokers and smokers. Values indicate means (standard deviations) and the number of subjects.

Appendix S4. Additional information from models based on IL1beta saliva: confidence intervals of the model parameters.

Appendix S5. Additional information about the performance measures: confidence intervals of the discrimination and classification parameters.

APPENDIX S1



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DITAME DO COMITÉ DE ÉTICA DA INVESTIGACIÓN DE SANTIAGO-LUGO

Juan Manuel Vázquez Lago, Secretario do Comité de Ética da Investigación de Santiago-Lugo

CERTIFICA:

Que este Comité avaliou na súa reunión do día 19/05/2015 o estudo:

Título: Asociación de la periodontitis crónica con las enfermedades sistémicas: análisis de la interacción microbioma-hospedador a nivel oral y sistémico
Promotor: Xunta de Galicia
Tipo de estudo: Outros
Versión:
Código do Promotor: EM 2014/025
Código de Rexistro: 2015/006

E, tomando en consideración as seguintes cuestións:

- A pertinencia do estudo, tendo en conta o coñecemento dispoñible, así coma os requisitos legais aplicables, e en particular a Lei 14/2007, de investigación biomédica, o Real Decreto 1716/2011, de 18 de novembro, polo que se establecen os requisitos básicos de autorización e funcionamento dos biobancos con fins de investigación biomédica e do tratamento das mostras biolóxicas de orixe humana, e se regula o funcionamento e organización do Rexistro Nacional de Biobancos para investigación biomédica, a ORDE SAS/3470/2009, de 16 de decembro, pola que se publican as Directrices sobre estudos Posautorización de Tipo Observacional para medicamentos de uso humano, e a Circular nº 07/2004, investigacións clínicas con produtos sanitarios.
- A idoneidade do protocolo en relación cos obxectivos do estudo, xustificación dos riscos e molestias previsibles para o suxeito, así coma os beneficios esperados.
- Os principios éticos da Declaración de Helsinki vixente.
- Os Procedementos Normalizados de Traballo do Comité.

Emite un **INFORME FAVORABLE** para a realización do estudo **polo/a investigador/a do centro:**

Centros	Investigadores Principais
USC; Facultade de Medicina e Odontoloxía	Inmaculada Tomás Carmona

En Santiago de Compostela, a 25 de maio de 2015
O secretario NOMBRE VAZQUEZ LAGO JUAN MANUEL - NIF 44829259M
Juan M. Vázquez Lago

APPENDIX S2

Section/Topic	Item		Checklist Item (TRIPOD Checklist)	Reported on Page
TITLE AND ABSTRACT				
Title	1	D;V	Identify the study as developing and/or validating a multivariable prediction model, the target population, and the outcome to be predicted	403
Abstract	2	D;V	Provide a summary of objectives, study design, setting, participants, sample size, predictors, outcome, statistical analysis, results, and conclusions	403, 404
INTRODUCTION				
Background and objectives	3a	D;V	Explain the medical context (including whether diagnostic or prognostic) and rationale for developing or validating the multivariable prediction model, including references to existing models	405, 406
	3b	D;V	Specify the objectives, including whether the study describes the development or validation of the model or both	406
METHODS				
Source of data	4a	D;V	Describe the study design or source of data (e.g., randomized trial, cohort, or registry data), separately for the development and validation data sets, if applicable	406
	4b	D;V	Specify the key study dates, including start of accrual; end of accrual; and, if applicable, end of follow-up	406
Participants	5a	D;V	Specify key elements of the study setting (e.g., primary care, secondary care, general population) including number and location of centres	406
	5b	D;V	Describe eligibility criteria for participants	406, 407
	5c	D;V	Give details of treatments received, if relevant	407
Outcome	6a	D;V	Clearly define the outcome that is predicted by the prediction model, including how and when assessed	409, 410, Figure 1
	6b	D;V	Report any actions to blind assessment of the outcome to be predicted	N/A
Predictors	7a	D;V	Clearly define all predictors used in developing the multivariable prediction model, including how and when they were measured	408, Figure 1
	7b	D;V	Report any actions to blind assessment of predictors for the outcome and other predictors	408
Sample size	8	D;V	Explain how the study size was arrived at	409
Missing data	9	D;V	Describe how missing data were handled (e.g., complete-case analysis, single	408

			imputation, multiple imputation) with details of any imputation method	
Statistical analysis methods	10a	D	Describe how predictors were handled in the analyses	409-412
	10b	D	Specify type of model, all model-building procedures (including any predictor selection), and method for internal validation	412
	10c	V	For validation, describe how the predictions were calculated	N/A
	10d	D;V	Specify all measures used to assess model performance and, if relevant, to compare multiple models	412, 413
	10e	V	Describe any model updating (e.g., recalibration) arising from the validation, if done	412, 413
Risk groups	11	D;V	Provide details on how risk groups were created, if done	409, 410
Development vs. validation	12	V	For validation, identify any differences from the development data in setting, eligibility criteria, outcome, and predictors	N/A
RESULTS				
Participants	13a	D;V	Describe the flow of participants through the study, including the number of participants with and without the outcome and, if applicable, a summary of the follow-up time. A diagram may be helpful	Figure 1
	13b	D;V	Describe the characteristics of the participants (basic demographics, clinical features, available predictors), including the number of participants with missing data for predictors and outcome	413, Tables 1 Appendix S3
	13c	V	For validation, show a comparison with the development data of the distribution of important variables (demographics, predictors and outcome)	N/A
Model development	14a	D	Specify the number of participants and outcome events in each analysis	Figure 1
	14b	D	If done, report the unadjusted association between each candidate predictor and outcome	415, 416, Figures 2
Model specification	15a	D	Present the full prediction model to allow predictions for individuals (i.e., all regression coefficients, and model intercept or baseline survival at a given time point)	418, Table 2, Appendix S4
	15b	D	Explain how to use the prediction model	419, Figures 3
Model performance	16	D;V	Report performance measures (with CIs) for the prediction model	422, 423, Table 3, Figures 4,

				Appendix S5
Model-updating	17	V	If done, report the results from any model updating (i.e., model specification, model performance)	N/A
DISCUSSION				
Limitations	18	D;V	Discuss any limitations of the study (such as nonrepresentative sample, few events per predictor, missing data)	430, 431
Interpretation	19a	V	For validation, discuss the results with reference to performance in the development data, and any other validation data	N/A
	19b	D;V	Give an overall interpretation of the results, considering objectives, limitations, results from similar studies, and other relevant evidence	427-430
Implications	20	D;V	Discuss the potential clinical use of the model and implications for future research	430, 431
OTHER INFORMATION				
Supplementary information	21	D;V	Provide information about the availability of supplementary resources, such as study protocol, Web calculator, and data sets	408 Appendix S1
Funding	22	D;V	Give the source of funding and the role of the funders for the present study	404

*Items relevant only to the development of a prediction model are denoted by D, items relating solely to a validation of a prediction model are denoted by V, and items relating to both are denoted D;V.

APPENDIX S3

Appendix S3.1

CLINICAL PARAMETERS	STUDY GROUPS (NON-SMOKERS)		
	Control group (n=40)	**Non-treated Perio group (n=36)**	***P* Value**
Age (years)	42.77 (10.53)	54.72 (9.79)	<0.001
Gender			
Male	22	18	NS
Female	18	18	
No. of teeth	27.12 (2.28)	24.58 (4.48)	0.007
Full mouth			
BPL (%)	19.32 (13.30)	54.91 (28.91)	<0.001
BOP (%)	9.15 (6.23)	55.66 (20.95)	<0.001
PPD (mm)	2.11 (0.39)	3.58 (0.78)	<0.001
CAL (mm)	1.21 (0.42)	3.57 (1.23)	<0.001
	Treated Perio group (n=30)	**Non-treated Perio group (n=30)**	***P* Value**
Age (years)	51.93 (8.48)		NA
Gender			
Male	12		NA
Female	18		
No. of teeth	23.46 (3.85)		NA
Full mouth			
BPL (%)	31.10 (19.72)	45.53 (27.66)	0.002
BOP (%)	30.96 (14.33)	52.53 (18.30)	<0.001
PPD (mm)	2.86 (0.54)	3.50 (0.66)	<0.001
CAL (mm)	3.09 (1.04)	3.30 (1.09)	NS

Values indicate means (standard deviations) and the number of subjects.

Appendix S3.2

CLINICAL PARAMETERS	STUDY GROUPS (SMOKERS)		
	Control group (n=22)	**Non-treated Perio group (n=43)**	***P* Value**
Age (years)	39.62 (9.20)	48.30 (8.92)	<0.001
Gender			
Male	10	20	NS
Female	22	23	
Cigarettes/day (no.)	8.68 (6.11)	16.92 (8.45)	<0.001
Months of smoking (no.)	215.00 (135.23)	343.14 (120.49)	0.002
No. of teeth	28.37 (1.66)	23.19 (5.63)	<0.001
Full mouth			
BPL (%)	18.81 (16.97)	52.52 (30.37)	<0.001
BOP (%)	8.00 (9.28)	47.10 (20.87)	<0.001
PPD (mm)	2.03 (0.38)	3.68 (0.61)	<0.001
CAL (mm)	1.12 (0.41)	3.77 (1.30)	<0.001
	Treated Perio group (n=20)	**Non-treated Perio group (n=20)**	***P* Value**
Age (years)	50.24 (8.53)		NA
Gender			
Male	10		NA
Female	10		
Cigarettes/day (no.)	19.20 (9.53)		NA
Months of smoking (no.)	344.65 (97.88)		NA
No. of teeth	20.70 (5.33)		NA
Full mouth			
BPL (%)	41.55 (19.19)	46.40 (30.85)	NS
BOP (%)	24.15 (12.16)	55.15 (23.72)	<0.001
PPD (mm)	2.89 (0.50)	3.55 (0.71)	<0.001
CAL (mm)	3.28 (1.61)	3.52 (1.66)	NS

Values indicate means (standard deviations) and the number of subjects.

APPENDIX S4

IL1beta-based Model (Untreated Periodontitis/ Periodontal Health)		Intercept 95% CIs	Variable 95% CIs
Non-Smoker	1.053 + 0.006 **IL1beta**	-1.699 -0.408	0.002 0.010
Smoker	0.0817 + 0.005 **IL1beta**	-0.587 0.751	0.0003 0.010
IL1beta-based Model (Untreated Periodontitis/ Treated Periodontitis)		**Intercept 95% CIs**	**Variable 95% CIs**
Non-Smoker	-0.690 + 0.004 **IL1beta**	-1.367 -0.013	0.001 0.007
Smoker	-0.794 + 0.018 **IL1beta**	-1.746 0.157	-0.002 0.039

APPENDIX S5

IL1beta Model (modelling 1)	AUC 95% CIs	ACC (%) 95% CIs	SENS (%) 95% CIs	SPEC (%) 95% CIs	PPV (%) 95% CIs	NPV (%) 95% CIs
Non-Smoker	0.734 0.925	68.4 86.8	58.3 86.1	70.0 92.5	67.5 90.6	67.3 86.4
Smoker	0.556 0.822	60.0 81.5	53.4 81.3	59.0 91.0	75.0 94.7	43.2 68.9
IL1beta Model (modelling 2)	**AUC 95% CIs**	**ACC (%) 95% CIs**	**SENS (%) 95% CIs**	**SPEC (%) 95% CIs**	**PPV (%) 95% CIs**	**NPV (%) 95% CIs**
Non-Smoker	0.528 0.813	58.3 80.0	33.3 70.0	73.3 96.6	64.0 95.0	56.0 74.3
Smoker	0.539 0.875	60.0 87.5	50.0 90.0	60.0 95.0	62.5 94.1	60.0 88.8

Modelling 1: first phase modelling consisting of untreated periodontitis/periodontal health. Modelling 2: second phase modelling consisting of untreated periodontitis/treated periodontitis.

ABBREVIATIONS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Glossary of Abbreviations

All abbreviations that have been used in this Thesis are listed here including abbreviations used only in figures/tables/appendices. Some of them were written in the plural, for which the corresponding abbreviation was incorporated an "s" in lower case.

8-PHdG: 8-hydroxideoxyguanosine

A. actinomycetemcomitans: *Aggregatibacter actinomycetemcomitans*

ACC: accuracy

ACPA: anti-citrullinated protein antibodies

AgP: aggressive periodontitis

ALP: alkaline phosphatase

AST: aspartate aminotransferase

AUC: area under the curve

bc: bias-corrected

BG: beta-glucuronidase

BL: bone loss

BOP: bleeding on probing

BPL bacterial plaque levels

C: control

CA: calcium

CAL: clinical attachment loss

CBA: cytometric bead array

CC: control condition

CCD: charge-coupled device

CGR: colourimetric griess reaction

CI: confidence interval

COL: collagenase

Colour: colourimetric

COPD: chronic obstructive pulmonary disease

CP: chronic periodontitis

CPITN/CPI: community periodontal index of treatment needs

CRP: C-reactive protein

csv: comma-separated values

Ctx: C-terminal cross-linked telopeptide of type I collagen

CVD: cardiovascular disease

Dev: deviation

DKK: dickkopf-related protein

DL: detection limit

DNA: deoxyribonucleic acid

DOI: digital object identifier

DOR: diagnostic odds ratio

DPP: dipeptidyl peptidase

DTA: diagnostic test accuracy

E: bacterial and host-derived enzymes and inhibitors

EGF: epidermal growth factor

ELISA: enzyme-linked immunosorbent assay

F. nucleatum: Fusobacterium nucleatum

Fluor: fluorimetric

FN: false negative

FNR: false negative ratio

FP: false positive

FPR: false positive rate

G: gingivitis

GAG: glycosaminoglycan

GCF: gingival crevicular fluid

Ge: generalised

GI: gingival index

GMCSF: granulocyte-macrophage colony-stimulating factor

H: health in periodontium

Hb: haemoglobin

HGF: hepatocyte growth factor

HMP: human microbiome project

HPLC: high performance liquid chromatography

HSROC: hierarchical summary ROC

HTS: high-throughput sequencing

I: inflammatory mediators and host-response

IC: immunochromatography

ICD: international classification of diseases

ICSBP: international consortium for salivary biomarkers of periodontitis

ICTP: cross-linked carboxyterminal telopeptide of type I collagen

ID: identity document

IFMA: immunofluorometric assay

IFN: interferon

Ig: immunoglobulin

IHMP: project of the integrated human microbiome

IL: interleukin

IQR: interquartile range

LDH: lactate dehydrogenase

LED: light-emitting diode

Lo: localised

LPS: lipopolysaccharide

LR-: negative likelihood ratio

LR+: positive likelihood ratio

M: moderate

MC: Markov chain

MCP1: monocyte chemoattractant protein-1

MFI: mean fluorescence intensity

Mi: mild

MIP: macrophage inflammatory protein

ml: milliliter

MMP: matrix metalloproteinase

MPO: myeloperoxidase

Multiplex cytometry: multiparametric cytometry

N: no

NA: not applicable

Ng: nanogram

NK: natural killer

NO: nitric oxide

NPV: negative predictive value

NS: not significant

NSp: not specified

Ntx: N-terminal cross-linked telopeptide of type I collagen

O: others

ON: osteonectin

OPG: osteoprotegerin

OR: odds ratio

OSM: oncostatin M

P: periodontitis

P. gingivalis: Porphyromonas gingivalis

P. intermedia: Prevotella intermedia

PA: plasminogen activator

PAF: platelet-activating factor

PAI: plasminogen activator inhibitor

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PDB: protein data bank

PDGF: platelet-derived growth factor

PE: phycoerythrin

PF: false positive

pg: picogram

PGE: prostaglandin E

PGF: prostaglandin F

pH: potential of hydrogen

PI: plaque index

PICO: patient, index test, comparison, outcome

PMID: pubmed identifier

PMN: polymorphonuclear neutrophil

POCID: point of care immunoflow device

PPD: probing pocket depth

PPV: positive predictive value

PRISMA: preferred reporting items for systematic reviews and meta-analysis

PROSPERO: international prospective register of systematic reviews

PTN: periostin

qPCR: quantitative PCR or real-time PCR

QUADAS: quality assessment of diagnostic accuracy studies

ra: receptor antagonist

RA: rheumatoid arthitis

RANKL: receptor-activator of nuclear factor-κB ligand

RANTES: regulated on activation, normal T cell expressed and secreted

RNA: ribonucleic acid

ROC: receiver operating characteristic

rRNA: ribosomal RNA

S: severe

SENS: sensitivity

SNP: single nucleotide polymorphism

SP: substance P

SPEC: specificity

SRP: scaling and root planning

STARD: standars for reporting of diagnostic accuracy studies

T: tissue-breakdown products and bone-remodelling molecules

T. denticola: Treponema denticola

T. forsythia: Tannarella forsythia

TC: target condition

TGF: transforming growth factor

Th: T helper

TIMP: tissue inhibitor of matrix metalloproteinase

TN: true negative

TNF: tumour necrosis factor

To: total

TP: true positive

TPR: true positive rate

TRAP: tartrate-resistant acid phosphatase

Treg: regulatory T cells

TRIPOD: transparent reporting of a multivariable prediction model for individual prognosis or diagnosis

txt: text

USA: United States of America

VEGF: vascular endothelial growth factor

WHO: World Health Organisation

WoS: web of sciences

Y: yes

Zymo: zymography

µl: microliter

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