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|----------------------------------------------------------|----------------------------------------------------|
| <b>RAD:</b> 11-116181- -4                                | <b>FECHA:</b> 2014-04-28 10:23:03                  |
| <b>DEP:</b> 2020 DIRECCION DE NUEVAS CREACIONES          | <b>EVE:</b> 379 FASE NACIONAL INCLUIDO CAPITULO II |
| <b>TRA:</b> 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II | <b>FOLIOS:</b> 9                                   |
| <b>ACT:</b> 519 PRESENTACION                             |                                                    |

Abogado(a)/Señor(a)  
JUAN PABLO CADENA SARMIENTO

|                |             |               |
|----------------|-------------|---------------|
| <b>Asunto:</b> | Radicación: | 11-116181- -4 |
|                | Trámite:    | 11            |
|                | Evento:     | 379           |
|                | Actuación:  | 519           |
|                | Oficio:     | 5158          |
|                | Folios:     | 9             |

|                                     |          |            |  |                               |          |
|-------------------------------------|----------|------------|--|-------------------------------|----------|
| Patente de Invención                | <b>X</b> |            |  | Patente de Modelo de Utilidad |          |
| Vía Nacional                        |          |            |  |                               |          |
| Vía PCT (Fase Nacional)             | <b>X</b> | Capítulo I |  | Capítulo II                   | <b>X</b> |
| No. Solicitud Internacional         |          |            |  |                               |          |
| Fecha de Presentación Internacional |          |            |  |                               |          |

|                   |                            |                    |
|-------------------|----------------------------|--------------------|
| Descripción:      | Radicación 11-116181-00000 | 08/Septiembre/2011 |
| Reivindicaciones: | Radicación 11-116181-00000 | 08/Septiembre/2011 |
|                   | Radicación 11-116181-00003 | 05/Diciembre/2013  |
| Figuras:          | Radicación 11-116181-00000 | 08/Septiembre/2011 |
| Prioridad:        | US2009039184               | 01/Abril/2009      |

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

De acuerdo con lo establecido en el numeral 1.2.2.2 del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales, por tanto, se estudiara la reivindicación 1.

El problema planteado en esta solicitud consiste en la necesidad de desarrollar un

método para el diagnóstico de enfermedad periodontal o un estado bucal saludable.

Para resolver este problema técnico la solicitud en estudio proporciona un kit para el diagnóstico de enfermedad periodontal o un estado bucal saludable a partir de la determinación de metabolitos en una muestra de fluido crevicular gingival.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 8/19 // 8/34 // 8/44.

#### **4. De la solicitud de patente**

##### **Reivindicaciones (Art 30 D 486, Art 22 PCT)**

La nueva reivindicación 1 no se ajusta a las prescripciones señaladas para el caso de los kits de partes relativos a invenciones de combinación por cuanto no corresponde a la asociación de unidades funcionales/composiciones que incluyen dos o más principios activos. Si bien podría llegar a considerarse en términos de un kit de diagnóstico, por la forma como se presenta no comporta una relación técnica de tipo estructural entre la tira que permite recolectar y detectar los metabolitos asociados a la enfermedad periodontal y las instrucciones de uso de la misma, más allá de la que correspondería a un método para el diagnóstico de enfermedades, el cual, como es bien sabido, forma parte de las excepciones a la patentabilidad mencionadas en el Art 20 lit d) D 486. Evidentemente la reivindicación presentada en términos de kit se refiere a un método de diagnóstico toda vez que implica los siguientes pasos secuenciales: (1) recolección de datos, (2) comparación de los datos frente a un valor normal, (3) identificación de cualquier desviación de los valores normales y (4) la toma de decisión respecto a la condición.

Un kit-de-partes referido a una invención de combinación está conformado por preparaciones que incluyen dos o más componentes individuales, donde tales componentes forman una unidad funcional. Un kit de diagnóstico y la presente cláusula hace referencia a un elemento tipo tira de recolección de fluidos junto con un instructivo para el diagnóstico de salud oral, lo cual no corresponde a la definición de un kit de partes. Se sugiere a la solicitante hacer las aclaraciones respectivas. Asimismo, se reitera a la Solicitante que las instrucciones no hacen parte de los elementos estructurales que definen un producto o un procedimiento.

##### **5. Descripción (Art 28 D 486)**

La descripción no divulga las características técnicas de las tiras para recolectar la muestra de fluido crevicular gingival de manera suficientemente completa para que la persona del oficio normalmente versada en la materia pueda ejecutarla, toda vez que no se incluye la descripción técnica de la composición química del material laminado, la disposición de dichas laminas, los reactivos dispuestos en dichas laminas para generar la reacción con los metabolitos presentes en el fluido u otras características químicas que permitan la detección y cuantificación de los metabolitos asociados a la enfermedad periodontal.

##### **6. Determinación del Estado de la Técnica**

El estado de la técnica se determinó con base en la fecha de presentación internacional: abril 1 de 2009, y se encontraron los siguientes documentos que

anticipan el objeto de esta solicitud.

| Ítem | Documento                                                          | Título                                                                                                               | Fecha de Publicación |
|------|--------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|----------------------|
| D4   | WO2005073721                                                       | "A test kit for detecting periodontal disease"                                                                       | 11/Agosto/2005       |
| D5   | Ozmeric Nurdan, Clinican Chimica Acta 343 (2004): 1-16             | "Advances in periodontal diseases markers"                                                                           | 2004                 |
| D6   | Hanioka, T. et al., Journal Periodontal Research 2005; 40; 331-338 | "Relationship between periodontal disease status and combination of biochemical assays of gingival crevicular fluid" | 2005                 |

El documento D4<sup>1</sup> divulga un kit y un método para el diagnóstico de enfermedades periodontales mediante el análisis de una muestra de la cavidad oral del paciente, donde dicho kit comprende un primer test que detecta una primera sustancia de origen bacteriano y un segundo test para la detección de una segunda sustancia relacionada con la respuesta inmune o inflamatoria del paciente (Pág. 5, línea 21 a Pág. 6, línea 24).

El documento D5 enseña una revisión de compuestos marcadores determinados en fluido crevicular gingival y saliva asociados con enfermedad periodontal. D2 revela un listado de marcadores clasificados como enzimas, proteínas, inmunoglobulinas y otros compuestos utilizados en el diagnóstico de la enfermedad periodontal (Pág. 1, resumen).

El documento D6 divulga un estudio clínico realizado sobre 27 voluntarios con el objetivo de evaluar marcadores bioquímicos en el fluido crevicular gingival como metodología de tamizaje para el diagnóstico de enfermedad periodontal (Pág. 331, resumen).

#### **Examen de Patentabilidad (Art 14 D486)**

#### **Nivel Inventivo (Art 18 D 486)**

#### **Reivindicación 1**

#### **Frente a D4 y D5**

La reivindicación 1 consiste en un kit para el cuidado oral usado para monitorear la enfermedad periodontal en un sujeto, donde el kit comprende: una o más tiras de recolección de fluido crevicular gingival para recolectar una muestra de fluido crevicular gingival y para recuperar los metabolitos contenidos en la muestra de fluido crevicular gingival; e instrucciones para el diagnóstico de la salud oral de un sujeto que comprende (a) recolectar una muestra de fluido crevicular gingival del sujeto, (b) detectar un primer nivel de al menos un metabolito en una primera muestra de fluido crevicular gingival recolectada en un primer momento, (c) detectar un segundo nivel de al menos un metabolito en una segunda muestra de fluido crevicular gingival recolectada en un segundo momento, (d) comparar el nivel del

1 [http://worldwide.espacenet.com/publicationDetails/biblio?CC=WO&NR=2005073721A1&KC=A1&FT=D&ND=3&date=20050811&DB=worldwide.espacenet.com&locale=en\\_EP](http://worldwide.espacenet.com/publicationDetails/biblio?CC=WO&NR=2005073721A1&KC=A1&FT=D&ND=3&date=20050811&DB=worldwide.espacenet.com&locale=en_EP)

primer metabolito detectado con el nivel del segundo metabolito detectado, en donde el nivel diferencial del segundo metabolito detectado con relación al nivel del primer metabolito detectado indica un cambio en la enfermedad periodontal del sujeto.

| Características esenciales                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | D4                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | D5                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Kit para el cuidado oral usado para monitorear la enfermedad periodontal en un sujeto, donde el kit comprende:                                                                                                                                                                                                                                                                                                                                                                                                                                        | un kit para el diagnóstico de enfermedades periodontales que comprende:                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | Compuestos marcadores determinados en fluido crevicular gingival y saliva asociados con el estatus de la enfermedad periodontal                                                                                                                                                                                                                                                                                                                                                                                         |
| una o más tiras de recolección de fluido crevicular gingival para recolectar una muestra de fluido crevicular gingival y para recuperar los metabolitos contenidos en la muestra de fluido crevicular gingival;                                                                                                                                                                                                                                                                                                                                       | Dos juegos de papel absorbente (3 unidades cada uno) para la recolección de una muestra de fluido sub-gingival para la recuperación y detección de un primer metabolito que corresponde a una proteasa de <i>Porphyromonas gingivalis</i> o <i>Bacteroides forsythus</i> y un segundo metabolito que corresponde a elastasa de neutrófilo humano y un contenedor para dicha muestra que incluye un buffer de dilución (Pág. 16, línea 11 a Pág. 18, línea 27)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | Donde los fluidos orales pueden ser recolectados con tiras de papel o micropipetas (Pág. 7, Col. 2, Párr. [004])                                                                                                                                                                                                                                                                                                                                                                                                        |
| instrucciones para el diagnóstico de la salud oral de un sujeto que comprende (a) recolectar una muestra de fluido crevicular gingival del sujeto, (b) detectar un primer nivel de al menos un metabolito en una primera muestra de fluido crevicular gingival recolectada en un primer momento, (c) detectar un segundo nivel de al menos un metabolito en una segunda muestra de fluido crevicular gingival recolectada en un segundo momento, (d) comparar el nivel del primer metabolito detectado con el nivel del segundo metabolito detectado, | Y un método para diagnosticar enfermedad periodontal que comprende: (a) la recolección de una muestra de fluido sub-gingival en un primer momento – determinación 1 – empleando dos juegos de papel absorbente (3 unidades); (b) recortar la parte que contiene la muestra de dicho papel y depositarla en un recipiente contenedor que incluye un buffer para la congelación de la muestra; (c) colocar la muestra en un buffer de ensayo que incluye un sustrato cromógeno para la cuantificación de los metabolitos por espectrofotometría; (d) establecer los niveles de un primer metabolito que corresponde a una proteasa de <i>Porphyromonas gingivalis</i> o <i>Bacteroides forsythus</i> y de un segundo metabolito que corresponde a elastasa de neutrófilo humano; (e) detectar los niveles del metabolito bacteriano y del huésped (elastasa) en un segundo (6 meses después) y tercer momento (6 meses después) (Pág. 16, línea 11 a Pág. 20, línea 7) | un método para diagnosticar enfermedad periodontal que comprende: (a) la recolección de una muestra de fluido oral (b) determinación de los niveles establecer los niveles de metabolitos tales como: 8-hidroxideoxiguanosina, urato, ascorbato, cortisol, nitrito, ácido hialurónico, condroitina sulfato, (Pág. 5, Tabla 1) hidroxiprolina, lipoxina A, queratina, glucosa, metilglioxal, ácido láctico, ácido propiónico, ácido butírico, compuestos azufrados volátiles, y glutatión (Pág. 5, Tabla 2) entre otros. |

| Características esenciales                                                                                                                                                     | D4                                                                                                                                                                                                                               | D5                                                                                                                                   |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|
| en donde el nivel diferencial del segundo metabolito detectado con relación al nivel del primer metabolito detectado indica un cambio en la enfermedad periodontal del sujeto. | En donde el nivel diferencial del primer y segundo metabolito detectado (elastasa > 2ng y arg-gingipianina >0.30 U) es empleado como predictor de progresión de la enfermedad periodontal (Pág. 20, línea 9 a Pág. 21, línea 12) | En donde el nivel de dicho metabolito detectado es empleado como marcador de la enfermedad periodontal (Pág. 8, Col. 1, Párr. [001]) |

El documento D4 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga un kit para el diagnóstico de enfermedades periodontales que comprende: dos juegos de papel absorbente (3 unidades cada uno) para la recolección de una muestra de fluido sub-gingival para la recuperación y detección de un primer metabolito que corresponde a una proteasa de *Porphyromonas gingivalis* o *Bacteroides forsythus* y un segundo metabolito que corresponde a elastasa de neutrofilo humano y un contenedor para dicha muestra que incluye un buffer de dilución (Pág. 16, línea 11 a Pág. 18, línea 27) y un método para el diagnóstico de progresión de enfermedad periodontal a partir de los niveles de dichos metabolitos en diferentes tiempos.

La diferencia entre la invención definida en la reivindicación 1 y las enseñanzas del documento D4 consiste en los metabolitos detectados, toda que D4 divulga la detección de un primer metabolito de origen bacteriano y un segundo metabolito que corresponde a elastasa humana.

El efecto técnico que se origina al diseñar un kit para el diagnóstico de enfermedad periodontal en una muestra de fluido crevicular gingival a partir de la determinación de un metabolito seleccionado de isoleucina, leucina, guanosina, ácido úrico, uridina, metionina, prolina, triptófano, ácido ascórbico, entre otros, no es evidente a partir de la descripción de la presente solicitud.

Por lo tanto, el problema técnico objetivo que pretende resolver la invención consiste en diseñar un kit alternativo a los conocidos para la detección en fluido crevicular gingival de metabolitos asociados a la enfermedad periodontal.

Sin embargo, el documento D5 divulga compuestos marcadores detectados en fluidos orales que son recuperados mediante tiras de papel o micropipetas empleados en el diagnóstico de enfermedad periodontal. Específicamente, el documento D5 menciona determinación de los niveles de metabolitos tales como: 8-hidroxideoxiguanosina, urato, ascorbato, cortisol, nitrito, ácido hialurónico, condroitina sulfato, (Pág. 5, Tabla 1) hidroxiprolina, lipoxina A, queratina, glucosa, metilglioxal, ácido láctico, ácido propiónico, ácido butírico, compuestos azufrados volátiles, y glutatión (Pág. 5, Tabla 2) empleados para diagnosticar enfermedad periodontal.

En consecuencia, la persona del oficio normalmente versada en la materia contaba con la sugerencia o motivación razonable para diseñar un kit que incluya una tira de papel absorbente para recuperar la muestra de la cavidad oral y detectar los niveles de compuestos provenientes del metabolismo de un patógeno o del huésped mediante una reacción colorimétrica como lo divulga el documento D4, empleando los compuestos marcadores de enfermedad periodontal tales como: 8-

hidroxideoxiguanosina, urato, ascorbato, cortisol, nitrito, ácido hialuronico, condroitina sulfato, (Pág. 5, Tabla 1) hidroxiprolina, lipoxina A, queratina, glucosa, metilglioxal, ácido láctico, ácido propionico, ácido butírico, compuestos azufrados volátiles, y glutatión (Pág. 5, Tabla 2) divulgados en el documento D5 para obtener un kit para el diagnóstico y progresión de la enfermedad periodontal, por lo cual, la reivindicación 1 se considera obvia.

### Frente a D6 y D5

La reivindicación 1 consiste en un kit para el cuidado oral usado para monitorear la enfermedad periodontal en un sujeto, donde el kit comprende: una o más tiras de recolección de fluido crevicular gingival para recolectar una muestra de fluido crevicular gingival y para recuperar los metabolitos contenidos en la muestra de fluido crevicular gingival; e instrucciones para el diagnóstico de la salud oral de un sujeto que comprende (a) recolectar una muestra de fluido crevicular gingival del sujeto, (b) detectar un primer nivel de al menos un metabolito en una primera muestra de fluido crevicular gingival recolectada en un primer momento, (c) detectar un segundo nivel de al menos un metabolito en una segunda muestra de fluido crevicular gingival recolectada en un segundo momento, (d) comparar el nivel del primer metabolito detectado con el nivel del segundo metabolito detectado, en donde el nivel diferencial del segundo metabolito detectado con relación al nivel del primer metabolito detectado indica un cambio en la enfermedad periodontal del sujeto.

| Características esenciales                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | D6                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | D5                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Kit para el cuidado oral usado para monitorear la enfermedad periodontal en un sujeto, donde el kit comprende:                                                                                                                                                                                                                                                                                                                                                                                                                                        | un método para el diagnóstico de enfermedades periodontales que comprende:                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | Compuestos marcadores determinados en fluido crevicular gingival y saliva asociados con el estatus de la enfermedad periodontal                                                                                                                                                                                                                                                                                                                                                                                         |
| una o más tiras de recolección de fluido crevicular gingival para recolectar una muestra de fluido crevicular gingival y para recuperar los metabolitos contenidos en la muestra de fluido crevicular gingival;                                                                                                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | Donde los fluidos orales pueden ser recolectados con tiras de papel o micropipetas (Pág. 7, Col. 2, Párr. [004])                                                                                                                                                                                                                                                                                                                                                                                                        |
| instrucciones para el diagnóstico de la salud oral de un sujeto que comprende (a) recolectar una muestra de fluido crevicular gingival del sujeto, (b) detectar un primer nivel de al menos un metabolito en una primera muestra de fluido crevicular gingival recolectada en un primer momento, (c) detectar un segundo nivel de al menos un metabolito en una segunda muestra de fluido crevicular gingival recolectada en un segundo momento, (d) comparar el nivel del primer metabolito detectado con el nivel del segundo metabolito detectado, | (a) recolectar una muestra de fluido crevicular gingival con una tira de recolección de fluido (Periopaper, Proflow, Inc.) insertando la tira en el área gingival hasta encontrar resistencia y durante un tiempo de 30 segundos; (b) remover la tira de papel y almacenarla en buffer Tris-HCl (pH 7.5) con BSA 0.1% y azida de sodio 0.1%; (c) eluir la muestra de la tira de papel y determinar los niveles de metabolitos de interés mediante ELISA tales como: hemoglobina, transferrina, albumina, anti-tripsina, fibronectina, IgA, IgG, IgM (Pág. 332, Col. 2 a Pág. | un método para diagnosticar enfermedad periodontal que comprende: (a) la recolección de una muestra de fluido oral (b) determinación de los niveles establecer los niveles de metabolitos tales como: 8-hidroxideoxiguanosina, urato, ascorbato, cortisol, nitrito, ácido hialuronico, condroitina sulfato, (Pág. 5, Tabla 1) hidroxiprolina, lipoxina A, queratina, glucosa, metilglioxal, ácido láctico, ácido propionico, ácido butírico, compuestos azufrados volátiles, y glutatión (Pág. 5, Tabla 2) entre otros. |

| Características esenciales                                                                                                                                                     | D6                                                                                                                                                                           | D5                                                                                                                                   |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|
|                                                                                                                                                                                | 333, Col. 2) entre otros.                                                                                                                                                    |                                                                                                                                      |
| en donde el nivel diferencial del segundo metabolito detectado con relación al nivel del primer metabolito detectado indica un cambio en la enfermedad periodontal del sujeto. | En donde el nivel de IgA y elastasa detectados en las muestras de fluido crevicular gingival es empleado como predictor de la enfermedad periodontal (Pág. 335, Col. 1 a 3). | En donde el nivel de dicho metabolito detectado es empleado como marcador de la enfermedad periodontal (Pág. 8, Col. 1, Párr. [001]) |

El documento D5 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga un método para el diagnóstico de enfermedades periodontales que comprende: (a) recolectar una muestra de fluido crevicular gingival con una tira de recolección de fluido (Periopaper, Proflow, Inc.) insertando la tira en el área gingival hasta encontrar resistencia y durante un tiempo de 30 segundos; (b) remover la tira de papel y almacenarla en buffer; (c) eluir la muestra de la tira de papel y determinar los niveles de metabolitos de interés mediante ELISA (Pág. 332, Col. 2 a Pág. 333, Col. 2).

La diferencia entre la invención definida en la reivindicación 1 y las enseñanzas del documento D5 consiste en los metabolitos detectados, toda que D5 divulga la detección de metabolitos tales como: hemoglobina, transferrina, albumina, anti-tripsina, fibronectina, IgA, IgG, IgM

El efecto técnico que se origina al diseñar un kit para el diagnóstico de enfermedad periodontal en una muestra de fluido crevicular gingival a partir de la determinación de un metabolito seleccionado de isoleucina, leucina, guanosina, ácido úrico, uridina, metionina, prolina, triptófano, ácido ascórbico, entre otros, no es evidente a partir de la descripción de la presente solicitud.

Por lo tanto, el problema técnico objetivo que pretende resolver la invención consiste en diseñar un kit y un método de diagnóstico alternativo a los conocidos para la detección en fluido crevicular gingival de metabolitos asociados a la enfermedad periodontal.

Sin embargo, el documento D5 divulga compuestos marcadores detectados en fluidos orales que son recuperados mediante tiras de papel o micropipetas empleados en el diagnóstico de enfermedad periodontal. Específicamente, el documento D5 menciona determinación de los niveles de metabolitos tales como: 8-hidroxideoxiguanosina, urato, ascorbato, cortisol, nitrito, ácido hialuronico, condroitina sulfato, (Pág. 5, Tabla 1) hidroxiprolina, lipoxina A, queratina, glucosa, metilglioxal, ácido láctico, ácido propionico, ácido butírico, compuestos azufrados volátiles, y glutatión (Pág. 5, Tabla 2) empleados para diagnosticar enfermedad periodontal.

En consecuencia, la persona del oficio normalmente versada en la materia contaba con la sugerencia o motivación razonable para diseñar un kit que incluya una tira de papel absorbente para recuperar una muestra de la cavidad oral y detectar los niveles de compuestos provenientes del huésped mediante una reacción tipo ELISA como lo divulga el documento D6, empleando los compuestos marcadores de enfermedad periodontal tales como: 8-hidroxideoxiguanosina, urato, ascorbato, cortisol, nitrito, ácido hialuronico, condroitina sulfato, (Pág. 5, Tabla 1) hidroxiprolina,

lipoxina A, queratina, glucosa, metilglioxal, ácido láctico, ácido propionico, ácido butírico, compuestos azufrados volátiles, y glutatión (Pág. 5, Tabla 2) divulgados en el documento D5 para obtener un kit para el diagnóstico y progresión de la enfermedad periodontal, por lo cual, la reivindicación 1 se considera obvia.

## 7. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

| Item | Documento                                                          | Reivindicaciones afectadas | Requisito que afecta |
|------|--------------------------------------------------------------------|----------------------------|----------------------|
| D4   | WO2005073721                                                       | 1                          | Nivel inventivo      |
| D5   | Ozmeric Nurdan, Clinican Chimica Acta 343 (2004): 1-16             | 1                          | Nivel inventivo      |
| D6   | Hanioka, T. et al., Journal Periodontal Research 2005; 40; 331–338 | 1                          | Nivel inventivo      |

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



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## Review

## Advances in periodontal disease markers

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**Abstract**

A computer-assisted medline search was conducted to find the relevant articles concerning the periodontal disease markers in gingival crevicular fluid (GCF) and saliva published during the 10-year period from 1993 to July 2003. This review suggests that certain diagnostic uses of saliva and GCF show promise. Although both fluids have been used to evaluate the risk for an individual to develop periodontal disease and to monitor of the host response to periodontal therapy, GCF has the chance of being closely approximated to the periodontal tissues where periodontal disease begins. The enzymes contributed to extracellular matrix (ECM) molecules and non-ECM molecules degradation and markers for polymorphonuclear leukocytes (PMN) activity and influx into the gingival tissue seem to provide valuable information regarding the periodontal disease diagnosis and prognosis. There is also an increasing evidence implicating reactive oxygen species and nitric oxide pathway in the pathogenesis of periodontal diseases. Although promising results have been achieved with the assays evaluating the markers in assessment of periodontal disease status, up to now, none of these tests are used routinely. Further, one commercially available genetic test has been reported to have the potential to be used to predict the periodontal disease, but there are controversial reports on this genetic susceptibility test.

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*Keywords:* Periodontal disease; Saliva; Gingival crevicular fluid; Biochemical markers; Immunological markers; Diagnosis**1. Introduction**

Periodontitis is a disease characterized by loss of connective tissue attachment and bone around the teeth in conjunction with the formation of periodontal pockets due to the apical migration of the junctional epithelium. Although bacteria are obviously the initiating agent in periodontitis, the complexity of the associated microflora and the critical role of the host in determining the outcome of the bacterial challenge cause difficulties in defining specific disease markers

in periodontal diseases. Periodontal disease progression is episodic in nature on a tooth site level; however, the risk of developing periodontal disease is principally patient-based rather than site-based [1].

Bacterial virulence factors either result directly in degradation of host tissues or cause the release of biologic mediators from host tissue cells that lead to host tissue destruction. Mediators produced as a part of the host response that contribute to tissue destruction include proteinases, cytokines and prostaglandins.

Another important class of molecules in tissue destruction is the variety of enzymes produced by periodontal microorganisms for instance, collagen-degrading enzymes, elastase-like enzyme, trypsin-like proteases, aminopeptidases and dipeptidylpeptidases.

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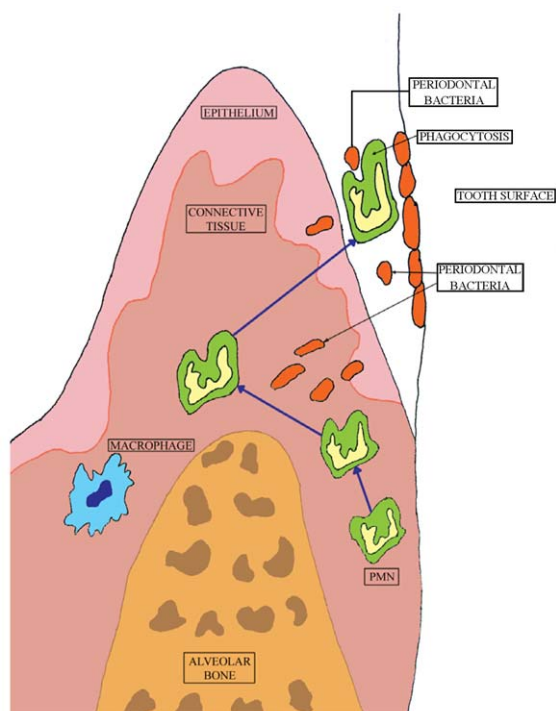


Fig. 1. A schematic presentation of periodontium and host responses towards periodontal bacteria in periodontal diseases. PMN: polymorphonuclear leukocytes, neutrophils.

Locally, bacterial lipopolysaccharide triggers monocytes, polymorphonuclear leukocytes (PMN, neutrophils), macrophages and the other cells to release interleukin-1 (IL-1), tumor necrosis factor (TNF) and prostaglandin  $E_2$  (PGE<sub>2</sub>) (Fig. 1). IL-1 and TNF appear to have an important role in periodontal tissue destruction and PGE<sub>2</sub> appears to be partly responsible for the bone loss associated with periodontitis [2] (Fig. 2).

Periodontal diseases have been seen at an increased rate in many countries throughout the world. Associated with this high prevalence of periodontal disease there has been an increase in tooth loss despite the availability of several new therapeutic methods and agents over the past two to three decades.

Clinical measurements used in the diagnosis of periodontal diseases are often of limited usefulness in that they are indications of previous periodontal disease rather than the present disease activity. Moreover, as various immunopathogenic mechanisms are involved in the disease process, the combinations of indicators are needed to improve the specificity of diagnosis.

Analysis of saliva and gingival crevicular fluid (GCF) may be especially beneficial in the determination of current periodontal status. Many studies have indicated that the determination of inflammatory me-

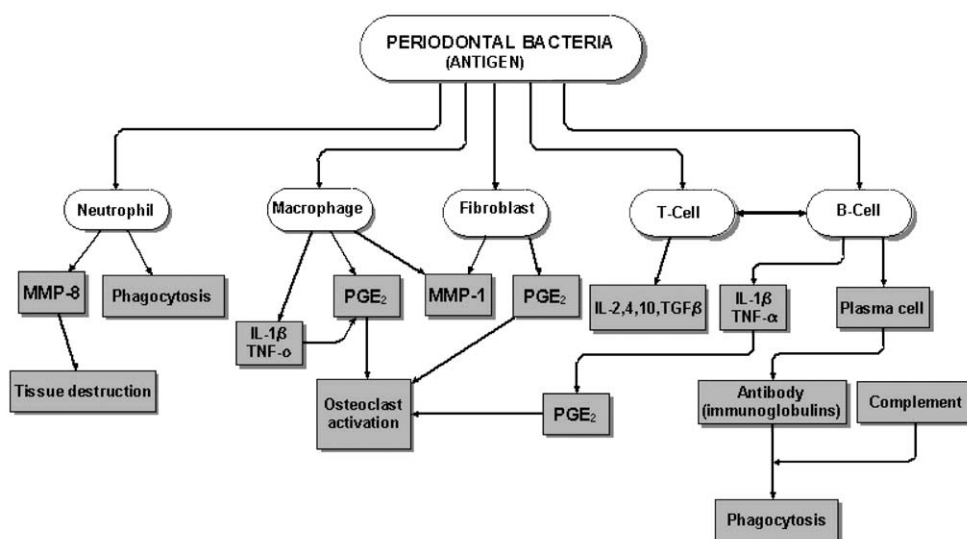


Fig. 2. Cells and their mediators that play roles in the periodontal disease pathogenesis. MMP: matrix metalloproteinase, IL-: interleukin, TGF-β: transforming growth factor β, TNF-α: tumor necrosis factor α, PGE<sub>2</sub>: prostaglandin  $E_2$ .

diator levels in biologic fluids is a good indicator of inflammatory activity. Therefore, studies related to the pathogenesis of periodontal diseases usually examine whether biochemical and immunological markers in saliva and/or GCF might be a marker to reflect the extent of periodontal destruction and to predict the future disease progression.

The aim of this article was to present a summary of data from the literature on periodontal disease markers especially in saliva and GCF. For this aim, we conducted a computer-assisted search on MEDLINE, using the key words “saliva and periodontal disease” and “gingival crevicular fluid and periodontal disease”. We reviewed human clinical studies on periodontal disease markers in saliva and GCF. Only studies performed on systemically healthy patients with periodontal disease were included in this review since systemic disease contribution to the periodontal health was considered to be another subject of review which might be of value in understanding the effect of systemic conditions on periodontal health and vice versa.

This manuscript reviews the current literature on host-derived and microbial-derived enzymes, non-enzymatic proteins, immunoglobulins, inflammatory mediators and cytokines that can be used for the prediction of periodontal disease and focuses on the GCF and saliva as potential sampling fluids of use.

## 2. Possible salivary markers for periodontal diseases

Long known primarily for its protective and lubricating properties, saliva has been examined to see whether it meets the demand for inexpensive and easy-to-use diagnostic aids for oral and systemic diseases. Due to the non-invasive and simple nature of saliva collection, saliva is also considered to be useful for the screening tests for periodontitis, and a means of monitoring the response to treatment. Salivary markers that have been studied for periodontal diagnosis include proteins of host origin (i.e., enzymes and immunoglobulins), phenotypic markers, host cells, hormones, bacteria and bacterial products, ions and volatile compounds [3]. In the view of the literature, recent findings on these markers in saliva are discussed below.

### 2.1. Enzymes

Saliva contains both host-derived and microbial-derived factors, including several enzymes that degrade proteins, proteoglycans, lipids and carbohydrates [4–8]. Enzymes in saliva can originate from cells in salivary glands, microorganisms, epithelial cells, PMN and can be derived from GCF. Enzymes, especially proteinases play an important role in the destruction of the periodontium, for instance, dipeptidylpeptidase IV (DPP IV) which contributes to the collagen degradation [9]. In a study conducted in our laboratory, DPP IV and alanine aminopeptidase (AAP) activity in whole saliva of periodontitis patients were found to be increased compared to the periodontally healthy controls [7]. AAP is a proteolytic enzyme that plays role in peptide hydrolysis [10]. It was found to be involved in degradation of collagen, which might further contribute to collagen degradation, carried out by DPP IV in periodontitis [7].

Neutrophils is an important cell type in host defense against periodontopathogenic bacteria and function to contain the microbial challenge through phagocytosis and killing and may contribute to local tissue changes by the release of tissue-degrading enzymes (Fig. 1). Neutrophil granules contain hydrolytic neutral enzymes, such as elastase, cathepsin G, myeloperoxidase and lysozyme, as well as hydrolases, including cathepsin B, cathepsin D and  $\beta$ -glucuronidase. Lactoferrin, neutrophil collagenase (matrix metalloproteinases-8, MMP-8) and MMP-9 are also stored in neutrophils' granules.

Analysis of  $\beta$ -glucuronidase in saliva may be an indicator of neutrophil influx into the gingival sulcus. Recently, Lamster et al. [11] found a significant association exists between periodontal clinical parameters, i.e., probing depth and salivary  $\beta$ -glucuronidase activity. In addition, total number of white blood cells and neutrophils in blood was observed to be correlated with the concentration of salivary  $\beta$ -glucuronidase. The salivary  $\beta$ -glucuronidase activity was found useful in predicting the presence of periodontal disease. Measures of increased influx of neutrophils and associated mediators such as  $\beta$ -glucuronidase in saliva may provide a measure of risk for patients with periodontal disease.

Aberrations of neutrophil function can lead to destruction of periodontal tissues since proteins such

as lysozyme, myeloperoxidase and lactoferrin act directly on the bacteria to prevent their growth and cause host tissue damage beside bacterial elimination. In a study, the longitudinal association of peroxidases, lysozyme and lactoferrin to the periodontal disease status was examined in peripheral blood neutrophils, GCF from diseased sites and paraffin-stimulated whole saliva. All enzyme concentrations were remarkably elevated in peripheral blood neutrophils, also reflected in their high concentrations in GCF. Although all these values were decreased with respect to healthy controls during the periodontal therapy, no similar longitudinal changes were seen in whole saliva except a decrease in salivary peroxidase concentrations [12].

Lactoferrin is the major iron-binding protein in saliva and provide non-specific innate host defenses. Although the mean salivary concentrations of lactoferrin were reported to be elevated in the saliva of periodontitis patients, it did not necessarily imply an increase in anti-microbial activity of saliva [13,14]. Groenink et al. [13] demonstrated a higher iron-saturation of lactoferrin in diseased subjects in comparison with the healthy subjects. Degradation products of iron-saturated lactoferrin create conditions that might induce tissue damage [15]. In a parallel report, localised aggressive periodontitis patients' whole saliva was reported to have higher lactoferrin levels than controls; however, their lactoferrin contained less iron. Lactoferrin was demonstrated to interact with *Actinobacillus actinomycetemcomitans*, which is a causative microorganism in aggressive periodontitis [16]. Therefore, colonization of *A. actinomycetemcomitans* may occur more readily in an environment containing lactoferrin with low iron levels and depressed level of iron found in lactoferrin may be resulted from both the iron-sequestering pathogenic bacteria and reduced capacity of lactoferrin to bind iron in the saliva of aggressive periodontitis patients [14].

Another neutrophil granule content, MMPs including neutrophil collagenase and serine proteinases seem to be also related to tissue destruction in periodontitis. MMP family in humans comprises 23 enzymes, which are involved in many biological processes and diseases. MMPs degrade most components of the extracellular matrix (ECM), as well as many non-ECM molecules. The major MMPs in neutrophils are MMP-8 and -9 and these are the main

collagen-degrading enzymes in saliva. Since MMPs can potentially cause tissue damage, their activity is controlled by the four members of the tissue inhibitor of metalloproteinase family (TIMP). The presence of MMPs in saliva has been studied comprehensively with the enzyme-linked immunosorbent assay (ELISA)-technique. The levels of MMP-1, MMP-3, MMP-8 and MMP-9, and their endogenous inhibitor, TIMP-1, in saliva of patients with adult periodontitis and localized juvenile periodontitis were evaluated. Elevated levels of MMP-8 were detected in saliva of adult periodontitis compared to localized juvenile periodontitis and the controls. Both MMP-1 and TIMP-1 were detected in all studied saliva samples, but not significant differences were detected between the studied groups [17]. In comparison to other assay systems such as immunoblotting, zymography and unlabeled collagen substrate, ELISA tests have several advantages including improved sensitivity, ease of use, moderately rapid throughput, the ability to quantify enzyme levels and the flexibility to test for multiple MMPs in single samples [18]. Therefore, not only detection of MMPs in saliva, but the identification of the other mediators can be easily performed with ELISA technique.

Nitric oxide (NO), which is a free radical with important cellular functions, is produced and released from human neutrophils and macrophages [19]. NO is synthesized from the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS). Arginase, which is an arginine-depleting enzyme, can compete with NOS for the common substrate L-arginine, and thus inhibit NO production. Recently, we investigated the salivary arginase activity in patients with periodontitis in order to elucidate the mechanism of its possible contribution to the disease process. We detected higher salivary arginase activity in periodontitis patients compared to healthy controls. The increased salivary arginase activity in periodontitis perhaps causing a decrease in NO synthesis also leads to a decrease in the antibacterial property of saliva and cause periodontal tissues to become more susceptible to existing pathogens [8]. According to the data showed the increase in synthesis of arginase, it may be concluded that the arginine–NO pathway is involved in the periodontal disease process.

Another enzyme, chitinase could play a role in the defense against chitin-containing oral pathogens. This

enzyme was observed significantly higher in the whole saliva of periodontitis patients than those in saliva from healthy controls. Periodontal treatment for a period of 5–6 months resulted in a three- to four-fold decrease in this enzyme activity [20]. In the same study, the activity of  $\beta$ -N-acetylhexosaminidase ( $\beta$ -NAH), which is another enzyme that hydrolyses glycosidic linkages, also decreased as a result of treatment, although to a lesser extent [20]. Other proteolytic enzymes that are produced by host cells and various oral organisms are also thought to be involved in the destruction of periodontal tissue (Table 1).

## 2.2. Non-enzymatic proteins and immunoglobulins

Non-enzymatic proteins have been examined in a number of studies to see whether there was a relation between periodontal disease and these proteins in saliva. For example, platelet-activating factor (PAF), a potent phospholipid inflammatory mediator, was identified in the mixed saliva of subjects with periodontal disease. Garito et al. [21] determined PAF activity in whole saliva of 69 untreated subjects and a significant correlation was reported between salivary PAF levels and the extent of periodontal disease. It was stated that this pro-inflammatory phospholipid may participate in the pathogenesis of periodontal

tissue injury and disease. In another study supported that hypothesis, a longitudinal evaluation of the effect of periodontal therapy on salivary PAF levels in chronic adult periodontitis patients was done and initial salivary PAF levels were found to be decreased following supragingival plaque control and further reduced following scaling and root planning [22].

The presence of vascular endothelial growth factor (VEGF), a multifunctional angiogenic cytokine, was reported for the first time in unstimulated saliva of patients with periodontal disease by Booth et al. [23]. VEGF was detected in all saliva samples and was significantly higher in patients than in healthy controls. This study suggested that VEGF could be relevant to angiogenic processes in healthy as well as diseased periodontal tissue and that the periodontal status influences the salivary level of VEGF [23].

Hepatocyte growth factor (HGF) acts as a mitogen, motogen, morphogen and anti-apoptotic factor for various kinds of epithelial cells. Ohshima et al. [24] examined whether HGF in whole saliva would be a useful marker for periodontal disease status. Significant correlations were reported between salivary HGF levels and the number of probing depths exceeding 4 mm, the number of probing depths exceeding 6 mm, the deepest probing depth of each subject and the percentage of sites positive for bleeding on probing.

Table 1  
Possible salivary markers for diagnosis of periodontal diseases

| Enzymes                     | Proteins                       | Immunoglobulins | Others                    |
|-----------------------------|--------------------------------|-----------------|---------------------------|
| Elastase [4]                | lactoferrin [12]               | IgA [93]        | PAF [21]                  |
| Amylase [5]                 | TIMP [17]                      | IgG [93]        | 8-OHdG [30]               |
| Dipeptidylpeptidase [7]     | VEGF [23]                      | IgM [93]        | urate [31]                |
| Alanine aminopeptidase [7]  | HGF [24]                       |                 | ascorbate [31]            |
| Arginase [8]                | fibronectin [25]               |                 | cortisol [94]             |
| $\beta$ -Glucuronidase [11] | albumin [26]                   |                 | nitrite [95]              |
| Myeloperoxidase [12]        | cystatins C, S, A, SN [27]     |                 | hyaluronic acid [96]      |
| Lysozyme [12]               | neopterin [34]                 |                 | chondroitin sulphate [97] |
| MMP-1 [17]                  | $\alpha$ -2-macroglobulin [88] |                 |                           |
| MMP-8 (collagenase-2) [17]  | $\alpha$ -1-antitrypsin [88]   |                 |                           |
| Chitinase [20]              | keratin [89]                   |                 |                           |
| Cathepsin G [88]            | C-reactive protein [90]        |                 |                           |
|                             | complement C3 [90]             |                 |                           |
|                             | IL-6 [90]                      |                 |                           |
|                             | EGF [91]                       |                 |                           |
|                             | defensin-1 [92]                |                 |                           |

MMP: matrix metalloproteinase, VEGF: vascular endothelial growth factor, HGF: hepatocyte growth factor, EGF: epithelial growth factor, 8-OHdG: 8-hydroxy-deoxyguanosine, IL-6: interleukin-6, TIMP: tissue inhibitor of matrix metalloproteinase, PAF: platelet-activating factor.

Fibronectin is a glycoprotein which mediates adhesion between cells. The consequent role of fibronectin is in the processes of growth, migration and differentiation of the cells. In a recent report, fibronectin in saliva was claimed to play a regulatory role in *Porphyromonas gingivalis* fimbria-mediated pathogenesis in adult periodontal disease [25]. Fimbriae was shown to be able to bind to fibronectin with high affinity. The ELISA test showed that the fibronectin contents in saliva of adult periodontal patients was significantly lower than that of healthy subjects [25].

Cystatins are physiological inhibitors of cysteine proteinases which are a group of proteolytic enzymes of neutrophils and widely distributed in human tissues and fluids. Up to now, 11 human cysteine proteases (cathepsins) have been identified. Exopeptidases (cathepsins B, C, H and X) and endopeptidases (such as cathepsins L, S, V and F) are involved in various biological and pathological processes and the control of these protease functions is carried out cystatins. In a recent study, subjects with periodontal disease have elevated levels of salivary cystatins compared to periodontally healthy individuals [26]. Cystatin C, cystatin S, cystatin A and cystatin SN could be detected in saliva samples [27]. Whole saliva of healthy subjects contained cystatin S, whereas cystatin C was barely detectable. In contrast, in saliva of gingivitis and periodontitis patients, both cystatins C and S levels were higher. The role for these proteins in periodontal disease is most likely protective. These findings were also supported by Henskens et al. [28]. They reported that levels of glandular derived proteins, amylase and cystatin C were significantly higher in whole and parotid saliva of subjects with periodontitis than in healthy controls. Smoking, another risk factor for periodontitis, is associated with lower cystatin activity and output of cystatin C during gingival inflammation [29].

As a result of stimulation by gram negative bacteria in periodontitis, neutrophils produce a high degree of the reactive oxygen species (ROS) via the respiratory burst as part of the host response to infection and an excess production leads an oxidative damage to gingival tissue, periodontal ligament and alveolar bone. In that way, ROS may involve in the pathogenesis of periodontal tissue destruction. ROS can cause tissue damage by a variety of different mechanisms, such as DNA damage, protein damage, oxidation of

important enzymes, stimulation of proinflammatory cytokine release and lipid peroxidation.

8-Hydroxy-deoxyguanosine (8-OHdG) is a product of oxidative DNA damage following specific enzymatic cleavage after 8-hydroxylation of the guanine and it is one of the most commonly used markers to evaluate this damage in a number of disorders including chronic inflammatory diseases. Takane et al. [30] collected saliva samples from 78 patients with untreated periodontitis and 17 healthy control subjects for determination of 8-OHdG with ELISA. The mean value of 8-OHdG in the saliva of periodontally diseased subjects was significantly higher than that of clinically healthy subjects. Salivary 8-OHdG levels decreased in response to periodontal therapy and approached the mean control values. These results were not so surprising since the strongest evidence for a role of ROS in the destruction of periodontal tissues comes from neutrophil infiltration against oral pathogenic bacterial invasion.

Periodontal diseases are probably associated with an imbalance between ROS and antioxidants in favor of the former due to the both an increase in free radical production and a defect in the total antioxidant activity of saliva. Urate is the predominant salivary antioxidant, with albumin and ascorbate providing minor contributions. Diab-Ladki et al. [31] found that, while the activity of the major antioxidants was not significantly reduced in saliva of patients with periodontitis, the total antioxidant activity and the total protein content of saliva was decreased. This suggests an equal contribution from all antioxidants in the defense mechanism against ROS production. It is likely that other components of saliva (e.g., salivary peroxidase and lactoferrin) also play a role in the protection of the oral tissues from ROS. In contrast to that report, the antioxidant potential of saliva was found not to be compromised in patients with periodontal disease, but the authors stated that this might relate to the antioxidant flow from the gingival crevicular fluid [32]. An improved understanding of the role antioxidants in periodontitis, and the influence of nutrition on antioxidant status, may lead to a possible nutritional strategy for the treatment of periodontal disease [33].

Neopterin, an actual molecular mass compound belonging to the class of pteridines, is an early and valuable biomarker of cellular immunity and released

from activated macrophages. Although there are few reports about neopterin activity in saliva, in a recent study of our group, higher GCF and salivary neopterin levels in aggressive periodontitis patients were detected [34]. However, there was not any difference existed in urinary neopterin levels between the patient and periodontal healthy groups. It was thought that the periodontal diseases stimulate the local immune response in patients and probably systemical response might not be involved in the disease process. We concluded that the neopterin levels in saliva might originate from neopterin in GCF due to the antigenic stimulation of oral bacteria [34]. Neopterin is known to take part in NOS production to produce NO and contributes to the natural antibacterial properties of saliva since it is a powerful antimicrobial substance produced in response to periodontopathogens and local inflammatory changes [35]. Though the exact source is still ambiguous, it was reported that NO in saliva might partly originate from cellular sources such as neurones, endothelial cells and macrophages [36]. Activated macrophages were shown to produce neopterin and this might also be valid for patients with periodontitis since macrophage infiltration and activation are known to be characteristics of chronic inflammation like periodontitis, provoked by pathogenic bacteria [37]. NO in saliva may be part of the non-specific natural defense mechanisms of the oral cavity against pathogenic bacteria.

In a parallel study, we evaluated the effect of periodontal treatment on neopterin profile in aggressive periodontitis [38]. A statistically significant difference in salivary neopterin levels was detected after the periodontal treatment compared to the control group without periodontal disease.

Although the inflammatory cellular infiltrate in periodontal tissues is predominated by neutrophils, however, B cells and plasma cells comprise a substantial proportion of the mononuclear cells infiltrate in periodontitis. These plasma cells are predominantly immunoglobulin G (IgG) with lower numbers of IgA cells in granulation tissues from periodontitis patients [39]. However, IgA is the predominant immunoglobulin in saliva. There are two subclasses of IgA: IgA1 and IgA2. IgA1 predominates in serum while IgA2 is found in higher concentrations in external secretions like saliva [2]. Hagewald et al. [40] investigated the humoral IgA response in the saliva of aggressive

periodontitis patients by measuring IgA subclasses and IgA antibodies reactive to microorganisms associated with periodontal disease. A statistically significantly lower concentration and secretion rate of total salivary IgA and IgA1 was found in the aggressive periodontitis group in saliva. It was stated that this reflects a general down-regulation of the humoral immune system in periodontal diseases.

Serum IgG, IgA and IgM and salivary IgA antibody levels reactive with extracts from, oral bacteria were measured before, during and after experimental gingivitis. A subgroup of participants with low mean numbers of bleeding gingival units after plaque accumulation showed significantly higher salivary IgA antibody levels reactive with *A. actinomycetemcomitans*, *Eubacterium saburreum* and *Streptococcus mutans*, as compared with the subgroup reaching high bleeding after probing scores. It was stated that high levels of salivary IgA directed against bacteria in dental plaque might thus protect against the development of gingivitis [41].

It is understood from all these studies that several mediators in saliva were mentioned in the pathogenesis of periodontal diseases. Since saliva is non-invasively and repeatedly sampled, it holds considerable promise as a biologic fluid that can be analyzed for diagnosis of periodontal diseases. Further, it can be collected by individuals without special training and can offer a cost-effective approach for the screening of large populations. However, most of the studies mentioned above were cross-sectional; therefore, it is difficult to interpret the significance of those few reports that examine levels of any particular marker. Well-controlled clinical trials conducted in large populations are necessary to confirm the importance of a specific diagnostic marker.

### 3. Possible markers in GCF for periodontal diseases

GCF is an inflammatory exudate that can be easy to collect by means of paper strips or micropipettes from gingival crevices of teeth. Host cells-derived inflammatory tissue response and bacterial products in GCF were assessed in several studies related to the markers of pathogenesis and diagnosis of periodontal disease. The cellular components of GCF are 70–80%

granulocytes, 10–20% monocytes/macrophages, 5% mast cells and 5% T lymphocytes. Most of the markers for periodontal diseases are originated from these cells in GCF. Both neutrophils and monocytes/macrophages are the essential parts of the host's inflammatory response in GCF. Neutrophil mediators are identified in GCF including leukotriene B<sub>4</sub> (LTB<sub>4</sub>), PAF, thromboxane B<sub>2</sub>, elastase and collagenase [41–45]. Monocytes/macrophages secrete mediators of inflammation, including PGE<sub>2</sub>, IL-1, IL-6, IL-8 and TNF [46–48]. Pro-inflammatory and anti-inflammatory cytokines are also identified in GCF [49,50]. Moreover, collagen breakdown products such as hydroxyproline and pyridinoline cross-linked carboxy-terminal telopeptide fragments of type I collagen are

found in GCF as an indicators of connective tissue catabolism for periodontal tissues [51,52] (Table 2).

### 3.1. Enzymes

Activity of neutrophil elastase has been implicated in periodontal tissue destruction in spite of the presence of potent proteinase inhibitors in GCF [53]. Buchmann et al. [54] analyzed myeloperoxidase,  $\beta$ -NAH and cathepsin D as indicators of the neutrophil associated host tissue destruction, and elastase- $\alpha$ -1-proteinase inhibitor complex ( $\alpha$ -1-EPI) as the major serum protein inactivating neutrophil elastase. The decrease in total myeloperoxidase,  $\beta$ -NAH and cathepsin D levels following periodontal therapy was associated with a

Table 2  
Possible gingival crevicular fluid markers for diagnosis of periodontal diseases

| Enzymes                          | Proteins                        | Immunoglobulins | Cytokine           | Others                           |
|----------------------------------|---------------------------------|-----------------|--------------------|----------------------------------|
| Lysozyme [12]                    | lactoferrin [12]                | IgA [124]       | VEGF [23]          | PAF [43]                         |
| MMP-8 (collagenase 2) [45]       | cystatins C, S [27]             | IgG [124]       | IL-1 $\beta$ [44]  | leukotriene B <sub>4</sub> [44]  |
| MMP-2 [63]                       | neopterin [34]                  | IgM [124]       | TNF- $\alpha$ [44] | thromboxane B <sub>2</sub> [44]  |
| MMP-9 [63]                       | $\beta$ -NAH [54]               | IgE [125]       | IL-2 [48]          | hydroxyproline [51]              |
| MMP-13 (collagenase-3) [64]      | TIMP [65]                       |                 | INF- $\alpha$ [49] | lipoxin A (4) [77]               |
| Neutral protease [98]            | osteopontin [71]                |                 | IL-10 [50]         | keratin [89]                     |
| Dipeptidylpeptidase [99]         | calprotectin [72]               |                 | RANTES [50]        | substance P [121]                |
| Alkaline phosphatase [100]       | hyaluronic acid [96]            |                 | IL-8 [85]          | PGE <sub>2</sub> [131]           |
| Aspartate aminotransferase [101] | chondroitin sulphate [109]      |                 | IL-1ra [126]       | glucose [132]                    |
| Myeloperoxidase [102]            | endothelin [110]                |                 | IL-4 [126]         | ICAM-1 [133]                     |
| Creatine kinase [103]            | proteoglycan [111]              |                 | IL-6 [127]         | methylglyoxal [134]              |
| Lactate dehydrogenase [103]      | thrombomodulin [112]            |                 | TGF- $\beta$ [128] | lactic acid [135]                |
| Elastase [104]                   | transferrin [113]               |                 | HGF [129]          | propionic acid [135]             |
| $\beta$ -Glucuronidase [105]     | C-reactive protein [113]        |                 | EGF [130]          | butyric acid [135]               |
| Cathepsin G, D, B [106]          | $\alpha$ -2-macroglobulin [113] |                 |                    | phyloquinone [136]               |
| Plasminogen [107]                | $\alpha$ -1-antitrypsin [113]   |                 |                    | volatile sulphur compounds [137] |
| Gingipain [108]                  | osteocalcin [114]               |                 |                    | glutathione [138]                |
|                                  | osteonectin [114]               |                 |                    | hydroxylslypyridinoline [139]    |
|                                  | hyaluronan [115]                |                 |                    |                                  |
|                                  | fibronectin [116]               |                 |                    |                                  |
|                                  | ICTP [117]                      |                 |                    |                                  |
|                                  | $\alpha$ -1-EPI [118]           |                 |                    |                                  |
|                                  | NTx [119]                       |                 |                    |                                  |
|                                  | E-selectin [120]                |                 |                    |                                  |
|                                  | neurokinin-A [121]              |                 |                    |                                  |
|                                  | MRP-8 [122]                     |                 |                    |                                  |
|                                  | calcitonin [123]                |                 |                    |                                  |
|                                  | albumin [124]                   |                 |                    |                                  |

MMP: matrix metalloproteinase, ICTP: pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen,  $\beta$ -NAH:  $\beta$ -N-acetyl-hexosaminidase,  $\alpha$ -1-EPI: elastase- $\alpha$ -1-proteinase inhibitor complex, TIMP: tissue inhibitor of matrix metalloproteinase, NTx: cross-linked N-telopeptide of type I collagen, E-selectin: endothelial leucocyte adhesion molecule, IL-: interleukin, IL-1ra: interleukin 1 receptor antagonist, TGF- $\beta$ : transforming growth factor  $\beta$ , IFN- $\alpha$ : interferon  $\alpha$ , TNF- $\alpha$ : tumor necrosis factor  $\alpha$ , VEGF: vascular endothelial growth factor, HGF: hepatocyte growth factor, EGF: epithelial growth factor, PGE<sub>2</sub>: prostaglandin E<sub>2</sub>, ICAM-1: intercellular adhesion molecule-1, PAF: platelet-activating factor.

decrease in  $\alpha$ -1-EPI [54]. Both concentration and total activity of myeloperoxidase in periodontally healthy subjects was reported to be lower than the periodontitis patients and a strong functional relativity existed between myeloperoxidase and elastase [55]. These results confirm the previous study stated that active neutrophil elastase and  $\alpha$ -1-EPI levels in healthy individuals were under the limit of detection of the spectrophotometric methods [53]. In a cross-sectional study of 117 subjects, myeloperoxidase and elastase were significantly correlated with periodontal attachment loss and probing depth [56]. Because of the large quantity of elastase available in neutrophils compared to the amount of elastase inhibitors in GCF, that at least locally transient free elastase occurs, which contributes to tissue destruction in chronic adult periodontitis [57]. It seems that data presentation by use of total activity of neutrophil enzymes compared to the concentration seems to be more sensitive in both the reflection of the actual enzymatic profile of GCF and also the existing clinical periodontal status [55].

As mentioned in previous sections, cysteine proteases (cathepsins) are also involved in neutrophil associated periodontal diseases. Cathepsin B and its endogenous inhibitors,  $\alpha$ -2-macroglobulin and cystatin were assayed in GCF. Total amounts of enzyme and inhibitor per GCF sample decreased after periodontal treatment and correlated positively with pocket depth and gingival, bleeding and plaque indices. Cystatin levels in GCF are considered to be insufficient to inhibit the enzyme substantially [58]. In a 2-year longitudinal study, the periodontal sites with rapid attachment loss were determined and cathepsin B levels at these sites were measured to be higher than non-attachment loss sites [59]. The increased cathepsin G content with increasing severity of periodontal inflammation in GCF from chronic adult periodontitis patients were also reported [60,61]. Cathepsin G may contribute to periodontal tissue destruction directly and indirectly, via proteolytic activation of latent neutrophil procollagenase (promatrix metalloproteinase-8) [61].

Some of the members of the matrix metalloproteinase family and the tissue inhibitors were also identified in GCF [62,63]. Chen et al. [45] reported the increased levels of active neutrophil collagenase in the GCF of periodontitis patients. The active forms of neutrophil type MMP-8 and MMP-13 in GCF were demonstrated to contribute to GCF collagenase activity

[64]. Many rather than single cellular sources of MMP in the diseased periodontium were identified in untreated periodontitis [64]. Another MMP, MMP-3 and TIMP-1 in GCF were evaluated as prognostic factors for the progression of periodontitis in 40 systemically healthy subjects over a 6-month period [65]. GCF was sampled from both healthy and diseased sites of each patient. The mean amounts of MMP-3 and TIMP-1 in diseased sites were significantly higher than in healthy sites. GCF levels of MMP-3 were highly correlated with clinical measurements taken at baseline, 3-month and 6-month visits. It was stated that sites with high GCF levels of MMP-3 and TIMP-1 were at significantly greater risk for progression of periodontitis [65].

Hanemaaijer et al. [66] developed monoclonal antibodies to MMP-8 that can be used in a chair-side dipstick test to monitor the course and treatment of periodontitis. Monoclonal and polyclonal antibody tests for MMP-8 was claimed to be coincided with the classical functional collagenase activity test from GCF in periodontal health and disease [67,68]. Moreover, assays used radiolabeled substrates to detect collagenase in whole-mouth GCF require several days to complete. To provide more rapid analyses of collagenase activity that are better adapted to clinical studies, a soluble biotinylated-collagen assay was developed and it was stated that this assay was specific, simple, rapid and reproducible and may facilitate diagnostic discrimination between stable and progressive lesions [69]. Bhide et al. [70] developed a fluorogenic septapeptide MMP assay to measure MMP activity with a fluorimeter and this assay was reported to provide estimates of inflammatory status, periodontal destruction, and response to treatment, especially in more severe periodontal lesions [70]. However, up to now, none of these tests are used routinely in daily practice.

### 3.2. Non-enzymatic proteins, inflammatory mediators and immunoglobulins

Extracellular matrix components including osteopontin (OPN), osteonectin and osteocalcin appear to be useful markers of bone resorption in periodontal diseases. OPN is a non-collagenous,  $\text{Ca}^{2+}$ -binding, glycosylated phosphoprotein in bone matrix and is produced by several cells including osteoblasts, osteoclasts and macrophages. Kido et al. [71] determined the OPN amount in GCF by ELISA and OPN level in

GCF was found to be increased with the progression of periodontal disease; however, when OPN amount was compared between the diseased and the healthy sites, non-significant difference was observed [71].

Calprotectin, a major cytosol protein of leukocytes, was identified in GCF by Kido et al. [72] and they found that the calprotectin concentration in GCF from patients with periodontitis was significantly higher than that in GCF from healthy subjects [72]. Expression of calprotectin from inflammatory cells appears to protect epithelial cells against binding and invasion by *P. gingivalis*. In periodontal disease, calprotectin may augment both the barrier protection and innate immune functions of the gingival epithelium to promote resistance to *P. gingivalis* infection [73]. In a study, significant positive correlations were observed in GCF calprotectin versus collagenase, AST levels and gingival index and calprotectin is thought to be a useful marker for periodontal inflammation [74]. Calprotectin in GCF was reported to be correlated with PGE<sub>2</sub> [72].

The arachidonic acid metabolites PGE<sub>2</sub> and LTB<sub>4</sub> are inflammatory mediators which are likely to be involved in the pathogenesis of periodontal disease. PGE<sub>2</sub> mediates vasodilatation, increases vascular permeability, enhances pain perception by bradykinin and histamine, alters connective tissue metabolism and enhances osteoclastic bone resorption. LTB<sub>4</sub> causes the accumulation of inflammatory cells in the inflamed sites and degranulation of polymorphonuclear leukocytes. The levels of PGE<sub>2</sub> were reported to correlate with the severity of the periodontal status, and the levels of LTB<sub>4</sub> correlate with gingival inflammation [75]. It was suggested that PGE<sub>2</sub> has considerable potential as a diagnostic marker for periodontal diseases and is predictive of future bone loss. Unfortunately, the current available test required to detect PGE<sub>2</sub> in gingival crevicular fluid is complex and not easy to perform as a chairside diagnostic test [76].

In the recent reports, the involvement of the inducible cyclooxygenase isoform (COX-2) and the role of novel lipid mediators in the pathogenesis of periodontal disease have been investigated [77]. COX catalyzes reactions that lead to the conversion of arachidonic acid to PGH<sub>2</sub> which is the common precursor of PGE<sub>2</sub>. Further, it was found that GCF samples from aggressive periodontitis patients contain PGE<sub>2</sub> and 5-LO-derived products, LTB<sub>4</sub>, and biosyn-

thesis interaction product, lipoxin (LXA<sub>4</sub>). LXA<sub>4</sub> can have a protective role in periodontitis, limiting further neutrophil recruitment and neutrophil-mediated tissue injury [78].

As it was clearly understood from the studies related with immun responses in periodontal diseases, not only cell-mediated but also humoral immun responses play an important role in periodontal diseases. Both IgA and IgG are present in GCF and are derived from serum and plasma cells in the gingival tissues. Recent studies of the relationship of antibody isotypes in GCF to progression of clinical attachment loss suggested that IgA in GCF has a protective function [79]. This may relate to the lack of complement activation by IgA. Alternately, the development of IgA antigen-specific responses are T-cell-dependent, and reductions in local levels of IgA may indicate a decrease in T-helper cell function. Specific IgG, IgA and IgM antibody titres to periodontopathogenic bacteria were measured in serum and gingival crevicular fluid [80]. Specific serum antibody titres were found to correlate with mean gingival crevicular fluid titres. Sites with high bleeding scores had significantly lower GCF IgG titres than those with less bleeding scores. These findings supported the concept that the humoral immune response is protective, as chronic periodontitis patients with greater pocket depths and more gingival inflammation had paradoxically lower antibody titres to suspected periodontopathogens [80].

### 3.3. Cytokines

Periodontal tissue cells stimulated by periodontal disease-associated bacteria, secreted inflammatory cytokines, including IL-1 $\beta$ , IL-8 and TNF- $\alpha$ , etc. Especially, in several studies, IL-1 $\beta$ , a potent stimulator of bone resorption, was implicated in the pathogenesis of periodontal tissue destruction [81]. IL-1 $\beta$  can modulate bone resorption by activating osteoclasts and stimulating PGE<sub>2</sub> synthesis [46]. The concentration of IL-1  $\beta$  was found significantly higher in patients with deep periodontal pockets than in patients with gingivitis. However, no significant differences were observed between deep and shallow periodontal pockets and this indicated that levels of IL-1 $\beta$  in GCF were increased in periodontitis regardless of the severity of disease [82]. These findings were supported by Champagne et al. [1] stated that both GCF

PGE<sub>2</sub> and IL-1 $\beta$  levels increased with time, in both stable and bone-loss sites. It was due to the sampling of stable sites in patients that do have progressing sites within their mouth. During the periods of disease progression, the release of inflammatory mediators increases all sites within the patient mouth, both at progressing and at stable sites. Although the disease progress differently at each local site because of site-specific characteristics, the host response is more global, and follows the same pattern at all sites within an individual's mouth [1,83].

In one of the studies dealing with the role of cytokines in the pathophysiological process of periodontal diseases and that was designed to quantitate IL-1 $\beta$  and a host-derived neutrophil activating peptide, IL-8, levels in GCF from patients with adult periodontitis, the total amount of IL-8 of the experimental group was found significantly higher than the control group. They suggested that the GCF IL-8 levels were influenced by local IL-1 $\beta$  activities and both of the cytokines exhibited dynamic changes upon severity of periodontal diseases [84]. We also conducted a study to evaluate the role of IL-8 in the pathogenesis of aggressive periodontitis and to determine the relationship between this cytokine and tissue destruction potential. For this aim, 24 patients with aggressive periodontitis and 14 systemically and periodontally healthy age-matched subjects were recruited for control group. The mean total amount and concentration of IL-8 was not found to be different significantly between the diseased group and healthy controls [85]. These findings were in contradiction with Chung et al.'s [86] data, who observed that periodontitis patients have demonstrated significantly higher total  $\beta$ -glucuronidase and lower IL-8 concentration. They speculated that the lower IL-8 concentration observed in patients with deeper mean probing depths, suggested an inverse relationship between IL-8 activity and neutrophil recruitment. Lower IL-8 concentrations were found positively correlated with elastase activity in patients co-infected with periodontopathogens [47].

#### 4. Conclusions

These data were collected to overview the strategies for identifying markers for patients with peri-

odontal disease based on the host response to the microbial structures in dental plaque. The next step should be to develop the non-invasive, cost-effective and easy-to-use diagnostic tests and analyses that are required for this approach to periodontal diagnosis with identification of these markers for active periodontal disease. Improved patient management should result from the incorporation of these tests into clinical practice. In recent years, different research groups developed biochemical and immunoreactive assays for MMP activity in GCF especially for discriminating the stable and progressive periodontal lesions. In the view of the literature, the assays for quantification of GCF MMP activity may provide improved assessment of periodontal disease status and response to treatment. Although promising results have been achieved with that assays, up to now, none of these tests are used routinely.

Further, one genetic test has been reported to have the potential to be used as a preventive measure, identifying people before they even show signs of the disease and getting them into early interventive treatment, most likely with a periodontist, however there are controversial reports on these commercially available genetic susceptibility test. It detects the simultaneous presence of a specific form of two interleukin genes allele 2 at the IL1A+4845 and IL1B+3954 loci. A fundamental premise of the test is that a combination of these alleles is responsible for increased secretion of IL- $\beta$ , which results in a hyper-inflammatory response to a bacterial challenge, thereby predisposing a person to develop severe chronic periodontitis. However, the relationship between specific IL-1 genotypes and the level of IL-1 $\beta$  in the gingival crevicular fluid was reported to be unclear. Similarly, the ability of the genetic susceptibility test to forecast which patients will develop increased bleeding on probing, periodontitis or loss of teeth or dental implants is ambiguous [87].

Since GCF has the chance of being closely approximated to the periodontal tissues where periodontal disease begins, it seems to provide more information than markers in saliva. The molecules in saliva can be also originated from salivary glands which cellular and biochemical mediators in saliva may reflect the diseases and metabolic status of glands instead of periodontal diseases. Because GCF constituents reflects the host response to the periodontopathogenic bacterial

antigens and the disease progression is essentially dependent upon the host response, evaluation of the markers in GCF is considered to be a good method in the determination of a person's risk for periodontal disease. Bacterial antigens triggers monocytes to release inflammatory mediators including PGE<sub>2</sub>, IL-1, IL-6, IL-8, TNF and collagenase that increase local destruction of the connective tissue structural elements; therefore, levels of these monocytic inflammatory mediators in GCF may well represent the markers of disease activity at a site level. Elevated GCF levels of neutrophil mediators including neutrophil elastase,  $\beta$ -glucuronidase and LTB<sub>4</sub> may reflect acute episodes of localised tissue destruction [1].

Most studies dealing with periodontal disease markers were conducted as cross-sectional study design. It is difficult to interpret the significance of these few cross-sectional reports that examine the levels of any particular marker in GCF or saliva. Therefore, longitudinal well-controlled clinical trials are needed to be performed in order to find the reliable marker(s) for periodontal diagnosis and to determine the risk of developing periodontitis.

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# Relationship between periodontal disease status and combination of biochemical assays of gingival crevicular fluid

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**Background:** Currently, no biochemical assay involving gingival crevicular fluid is utilized routinely as a screening test for periodontal disease.

**Objective:** The objective of the present study was to evaluate the potential of gingival crevicular fluid assay as a screening methodology.

**Methods:** The subject population was comprised of 27 volunteers. Nine participants were classified as 'subject with periodontal destruction' (SPD) exhibiting at least one site with pocket depth and attachment loss > 3.5 mm, whereas the remaining individuals were categorized as 'subject with minimal periodontal destruction' (SMD). Gingival crevicular fluid was collected from fixed sites via a standardized method. Biochemical assays of 12 substances (hemoglobin, albumin, transferrin,  $\alpha_1$ -antitrypsin, fibronectin, IgA, IgG, IgM, lactoferrin, myeloperoxidase and neutrophil elastase) were conducted at a commercial laboratory. Power transformation of total quantities in gingival crevicular fluid was performed for statistical analysis.

**Results:** Relationships between total quantity of each substance and periodontal disease status were unclear. Logistic regression analysis yielded six predictive models, which consisted of substance pairs: neutrophil elastase/IgA, neutrophil elastase/hemoglobin, neutrophil elastase/ $\alpha_1$ -antitrypsin and neutrophil elastase/IgG, and IgA/albumin and IgA/transferrin ( $p < 0.05$ ). Regression lines for SPD and SMD on a scattergram of IgA and neutrophil elastase were nearly parallel within the range of amounts in gingival crevicular fluid. The predictive model derived from both substances afforded sensitivity and specificity of 88% and 94%, respectively.

**Conclusions:** These results indicated that the combination of IgA and neutrophil elastase in gingival crevicular fluid may be crucial for prediction of periodontal disease status. Furthermore, these data suggested that biochemical assays employing both substances in gingival crevicular fluid may provide a satisfactory screening test for periodontal disease.

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Periodontal disease is characterized by chronic inflammatory lesions and destruction of supportive periodontal tissue. Subjective symptoms are typically mild during progression of the disease. In the early phase of periodontal disease, subjects tend to ignore the condition until more severe symptoms appear, for example, increased tooth mobility. Periodontal disease may be a major cause of tooth loss. In Japan, periodontal disease and dental caries display similar significance in terms of reason for tooth extraction (1). Accumulation of tooth loss leads to impairment of essential functions in quality of later life, i.e. conversation and eating. Thus, early detection of periodontal disease may be important with respect to quality of later life.

Gingival crevicular fluid and saliva contain substances associated with the host response, which could reflect periodontal disease status; therefore, determination of biochemical substances in oral fluid may be appropriate for early detection of periodontal disease (2, 3). Saliva is secreted from salivary glands; consequently, basic components may reflect a general condition rather than oral disease status. Salivary cotinine, a metabolite of nicotine, is utilized routinely as an indicator of current smoking (4). In lieu of crude saliva, whole saliva may include substances derived from various sites in the oral cavity. A few substances in whole saliva were correlated with periodontal disease: beta-glucuronidase activity (5), IgA1 (6) and elastase (7). Hemoglobin in whole saliva was associated with gingival inflammation (8, 9).

Several studies suggested the availability of assays employing saliva for early detection of periodontal disease; however, none of these tests is, at present, applied routinely (3). Gingival crevicular fluid contains informative substances close to periodontal tissue; as a result, gingival crevicular fluid may be a more suitable screening test sample in comparison with saliva. However, several barriers to routine utilization of gingival crevicular fluid screening methodology exist. Samples, which are collected from a single site in the mouth, may not reflect the whole

mouth status of the subject. Cost-effectiveness of bulk biochemical assays could also be an important consideration in terms of widespread application of this screening approach. In general, reagents essential for the assay of special substances may be expensive. A model capable of predicting periodontal disease activity using gingival crevicular fluid was developed (10). However, this model, which requires assessment of six substances, necessitates special assays.

Inexpensive assays of peripheral blood and urine specimens are performed routinely in bulk by commercial laboratories designed for general-health examination of large populations. The examination evaluates individual health status via a combination of several assays. Therefore, combination of those biochemical assays commonly applied to specimens of peripheral blood and urine could also be applied to gingival crevicular fluid samples. The objectives of the present investigation were to examine relationships between periodontal disease status and combination of biochemical assays with respect to gingival crevicular fluid samples and to evaluate a predictive model pertaining to subjects with periodontal disease.

## Material and methods

The current study deals with a screening test; as a result, subjects were selected from the general population. Participants consisted of 27 volunteers (15 males and 12 females,  $41.8 \pm 11.0$  years of age, average  $\pm$  SD). Subjects were office personnel employed by a business in Kyoto prefecture, Japan. The protocol, which was designed within the guidelines of the Declaration of Helsinki of the World Medical Association, was approved by the company's occupational safety and health committee. Informed consent was obtained from all participants prior to the study.

Collection of gingival crevicular fluid and periodontal assessments were performed on a portable bed under sufficient light provided by a light apparatus (Cold Light Supply, Nagashima Inc., Tokyo, Japan) at the

workplace. Gingival crevicular fluid samples were collected with a paper strip at three fixed sites per subject: mesio-palatal line angles of the upper maxillary first molars on both sides and at the deepest pocket within the mouth. The deepest pocket was identified in advance during a pilot examination 3 months prior to this investigation. Prior to collection of gingival crevicular fluid, supragingival plaque was removed with a cotton ball. A paper strip (Periopaper, Proflow Inc., Amityville, NY, USA) was inserted into the gingival crevice until resistance was encountered; it remained in place for 30 s. The paper strip was removed and stored in a sampling tube containing 1 ml of 0.05 M Tris-HCl buffer (pH 7.5) with 0.1% bovine serum albumin (Serological Proteins Inc., Kankakee, IL, USA) and 0.1% sodium azide (Nacalai Tesque, Kyoto, Japan) as preservative. The samples were then delivered to a commercial laboratory for the assays.

Following collection of gingival crevicular fluid, clinical assessments were conducted: pocket depth and attachment loss were measured at the mesial surfaces of the labial/buccal and lingual/palatal aspects of all existing teeth, with the exception of the third molars, with a pressure-sensitive probe (Vivacare TPS Probe, Vivadent, Schaun, Lichtenstein). Supragingival plaque and gingival inflammation were assessed with the plaque index (11) and the modified gingival index (12), respectively. Generally, screening tests are utilized for early detection of non-apparent disease, whereas dichotomous classification such as 'negative' and 'positive' functions in the tests to distinguish corresponding disease status. Therefore, in the present study, two categories, 'subject with periodontal destruction (SPD)' and 'subject with minimal periodontal destruction (SMD)' were employed, despite the general utility of three categories, namely, healthy, gingivitis and periodontitis, for periodontal diseases in the clinical setting. The periodontal disease category was defined on the basis of pocket depth and attachment loss dichotomy. Individuals demonstrating at least one site at which both pocket

depth and attachment loss exceeded 3.5 mm were defined as SPD. Remaining subjects, including gingivitis subjects, were defined as SMD.

At the commercial laboratory, elution of constituents from the paper strips was performed with 1 ml of samples by gentle agitation for 30 min. Gingival crevicular fluid samples were diluted for the multiple assays of 12 substances. Hemoglobin, albumin, transferrin,  $\alpha_1$ -antitrypsin, fibronectin, IgA, IgG, IgM, lactoferrin, myeloperoxidase and neutrophil elastase were assessed by sandwich enzyme-linked immunosorbent assays (13, 14). Anti-human polyclonal antibodies of hemoglobin, albumin transferrin,  $\alpha_1$ -antitrypsin IgA, IgG, IgM, myeloperoxidase (preceding seven acquired from Dakopatts, Glostrup, Denmark), fibronectin, neutrophil elastase and lysozyme (preceding three acquired from Serotec, Oxford, England) were introduced to the wells of 96-well microplates and absorbance was measured spectroscopically (Multiscan MS UV, Labsystems, Helsinki, Finland). The analyzer, which displayed sufficient sensitivity so as to permit detection of substances, required a 0.05-ml aliquot of sample in each assay for total amount of proteins in gingival crevicular fluid. Assay of lactoferrin was completed with a commercially available kit (Shionogi & Co., Ltd, Osaka, Japan). These assays were performed according to the manufacturer's recommended protocols.

Screening tests were evaluated with respect to sensitivity and specificity (15). Screening tests, which accurately identify individuals presenting with disease as well as disease-free subjects, are usually required. Sensitivity is the proportion of persons with active disease identified as positive by the screening test. Specificity is the proportion of persons without active disease correctly identified by the screening test. The probability of sensitivity or specificity ranges from 0 to 1. Probabilities approaching 1 result in categorization of a test as either highly sensitive or highly specific.

Total amount of each substance in the sample was enumerated. Distribu-

tion of gingival crevicular fluid sample quantities was usually skewed to lower values; therefore, data were transformed according to the established formula;  $X = ((x - a)^p - 1)/p$ . If  $p = 0$ ,  $X = \log(x - a)$ . Differences between SPD and SMD in terms of total amount in gingival crevicular fluid were examined with the Mann-Whitney test. Multiple logistic regression analysis was employed to develop a predictive model of periodontal disease status (SPD or SMD). Quantities of the substances in the sample served as independent variables in the backward stepwise elimination method. Statistical analyses including the power transformation were conducted with computer software (STATFLEX, Artech, Osaka, Japan). The level of significance was set at 5%.

## Results

Following clinical assessment, nine (six males and three females) and 18 subjects were identified as SPD and SMD, respectively (Table 1). Mean values of the plaque index and the modified gingival index were similar between groups: 1.1 for plaque index and 0.9

for modified gingival index ( $p > 0.05$ ). Pocket depth and attachment loss in SPD were significantly higher than corresponding parameters in SMD ( $p = 0.0152$  and  $0.0024$ , respectively). Mean values of pocket depth and attachment loss were calculated at two fixed sites where gingival crevicular fluid samples were collected. Pocket depth was  $2.5 \pm 0.6$  mm for SMD and  $3.2 \pm 1.0$  mm for SPD, whereas attachment loss values were  $0.5 \pm 0.9$  mm and  $2.4 \pm 1.7$  mm, respectively. The values at fixed sites were higher than the mean values of the whole mouth. Differences between SMD and SPD were significant in pocket depth and attachment loss ( $p = 0.0367$  and  $p = 0.0096$ , respectively).

Descriptive statistics of total quantity of 12 substances in gingival crevicular fluid are presented in Table 2. Mean value of total amount exceeded median value for all substances. For hemoglobin, albumin, transferrin and fibronectin, mean value of total amount exceeded the 75th percentile. Distributions of total amount were apparently skewed to lower levels for all substances (data not shown).

Table 1. Comparisons of clinical indices between subject with minimal periodontal destruction (SMD) and subject with periodontal destruction (SPD) groups

| Clinical indices        | All ( $N = 27$ ) | SMD ( $N = 18$ ) | SPD ( $N = 9$ ) | $p$    |
|-------------------------|------------------|------------------|-----------------|--------|
| Plaque index            | $1.1 \pm 0.7$    | $1.1 \pm 0.8$    | $1.1 \pm 0.7$   | 0.9211 |
| Modified gingival index | $0.9 \pm 0.6$    | $0.9 \pm 0.7$    | $0.9 \pm 0.6$   | 0.9208 |
| Pocket depth (mm)       | $2.2 \pm 0.4$    | $2.1 \pm 0.4$    | $2.5 \pm 0.5$   | 0.0152 |
| Attachment level (mm)   | $0.4 \pm 0.5$    | $0.2 \pm 0.4$    | $0.8 \pm 0.5$   | 0.0024 |

Table 2. Total amounts of biological markers in gingival crevicular fluid samples from 81 sites in 27 subjects

| Biological marker                  | Mean $\pm$ SD     | Median | IQR <sup>a</sup> |
|------------------------------------|-------------------|--------|------------------|
| Hemoglobin ( $\mu$ g)              | $7.0 \pm 26.4$    | 0.4    | 0–3.1            |
| Albumin ( $\mu$ g)                 | $13.9 \pm 26.7$   | 3.1    | 2.1–10.7         |
| Transferrin ( $\mu$ g)             | $0.55 \pm 0.63$   | 0.34   | 0.22–0.53        |
| $\alpha_1$ -antitrypsin ( $\mu$ g) | $0.72 \pm 0.62$   | 0.57   | 0.26–1.01        |
| IgA ( $\mu$ g)                     | $0.39 \pm 0.31$   | 0.28   | 0.17–0.52        |
| IgG ( $\mu$ g)                     | $2.5 \pm 2.4$     | 1.5    | 1.1–3.1          |
| IgM ( $\mu$ g)                     | $0.14 \pm 0.21$   | 0.06   | 0.02–0.14        |
| Myeloperoxidase (ng)               | $86.0 \pm 64.0$   | 71.6   | 39.2–114.3       |
| Fibronectin (ng)                   | $115.9 \pm 183.3$ | 47.4   | 23.9–104.3       |
| Neutrophil elastase (ng)           | $40.0 \pm 40.0$   | 24.8   | 15.7–48.4        |
| Lysozyme (ng)                      | $53.1 \pm 49.1$   | 36.5   | 20.7–73.9        |
| Lactoferrin (ng)                   | $150.9 \pm 133.7$ | 113.2  | 50.5–186.7       |

<sup>a</sup>IQR, inter-quartile range (25th percentile–75th percentile).

Table 3. Comparisons of total amount of biological markers in gingival crevicular fluid on a site basis between subject with minimal periodontal destruction (SMD) and subject with periodontal destruction (SPD)

| Biological marker                | SMD ( <i>N</i> = 54) |                  | SPD ( <i>N</i> = 27) |                  |
|----------------------------------|----------------------|------------------|----------------------|------------------|
|                                  | Median               | IQR <sup>a</sup> | Median               | IQR <sup>a</sup> |
| Hemoglobin (μg)                  | 0.3                  | 0–2.5            | 0.4                  | 0–4.7            |
| Albumin (μg)                     | 3.2                  | 2.4–10.6         | 2.7                  | 1.8–14           |
| Transferrin (μg)                 | 0.33                 | 0.25–0.48        | 0.37                 | 0.13–0.86        |
| α <sub>1</sub> -antitrypsin (μg) | 0.49                 | 0.27–0.89        | 0.74                 | 0.23–1.21        |
| IgA (μg)                         | 0.24                 | 0.16–0.45        | 0.43                 | 0.21–0.77        |
| IgG (μg)                         | 1.4                  | 1.1–3.1          | 1.8                  | 0.9–3.1          |
| IgM (μg)                         | 0.07                 | 0.02–0.15        | 0.05                 | 0.02–0.14        |
| Myeloperoxidase (ng)             | 71.6                 | 33.6–120.8       | 79.5                 | 42.5–113.3       |
| Fibronectin (ng)                 | 49.5                 | 19.2–78.7        | 47.1                 | 25.2–159.4       |
| Neutrophil elastase (ng)         | 28.8                 | 16.3–50.3        | 20.1                 | 8.7–43.8         |
| Lysozyme (ng)                    | 40.1                 | 19.6–75.2        | 34.4                 | 23.3–57.3        |
| Lactoferrin (ng)                 | 118.6                | 48.4–211.5       | 104.0                | 52.7–161.4       |

<sup>a</sup>IQR, inter-quartile range.

Significant differences were not detected for any substance.

Total amount of each substance was compared between the SPD and SMD groups on a site basis (Table 3). SPD was greater than SMD with respect to median values of hemoglobin, transferrin, α<sub>1</sub>-antitrypsin, IgA, IgG and myeloperoxidase. However, no significant difference was observed between SMD and SPD for any substance.

Periodontal disease status was predicted by multiple logistic regression analysis. Transformed amounts served as independent variables. Analyzed samples were utilized to determine whether any combinations of biochemical substances in gingival crevicular fluid were predictive of periodontal disease status. The deepest site was identified in advance following periodontal examination; therefore, data obtained from the deepest site was excluded from this model. The periodontal examination is itself definitive for periodontal disease status. Regression analyses were performed

exclusively with data derived from maxillary first molars. These analyses yielded six predictive models (Table 4). All models consisted of two significant variables (*p* < 0.05). Neutrophil elastase was included in four models, whereas IgA was a component of three models. Albumin, transferrin, hemoglobin, α<sub>1</sub>-antitrypsin and IgG were included in each model.

Total amounts of two substances, which were significant for that specific model, were plotted on a scattergram; subsequently, regression lines for SPD and SMD were generated separately (Fig. 1). Regression lines for SPD and SMD crossed within the range of quantities detected in five models (Figs 1A, B, D, E and F). Regression lines were nearly parallel on the scattergram of neutrophil elastase and IgA (Fig. 1C).

On the scattergram of neutrophil elastase and IgA (Fig. 1C), two points, which represent gingival crevicular

fluid samples from the same subject, were connected with a line (Fig. 2). Most of the lines were generated in a direction similar to that of the regression lines depicted in Fig. 1(C). Lines, which were directed perpendicular to regression lines, tended to be short, i.e. similar quantities were obtained from both sites in the same subject.

Quantities of neutrophil elastase and IgA were represented by higher values in the samples for each participant in order to develop a practical predictive model of periodontal disease status employing two samples of gingival crevicular fluid from fixed sites. The representative values of both substances in SPD and SMD were then plotted on a scattergram (Fig. 3). Regression lines were, again, parallel. Plots of either SPD or SMD were located close to each line. Most plots for SPD were located beneath those for SMD. This trend was consistent in the range of approximately 100-fold of the amount in gingival crevicular fluid: 0.02–2.0 μg for IgA and 1–100 ng for neutrophil elastase.

Predicted probabilities of SPD and SMD were enumerated in the multiple logistic regression analysis using the pair of representative values. Total amounts of IgA and neutrophil elastase were compared between SPD and SMD (Figs 4A and B, respectively). Predicted values were also compared between SPD and SMD (Fig. 4C). The plots in both groups overlapped with respect to IgA and neutrophil elastase; predicted values provided clearer separation of both groups. When an approximate cut-off point was defined, sensitivity and specificity of the test were 88% and 94%, respectively.

## Discussion

The ultimate goal of the present investigation was to examine evidence regarding the potential utilization of gingival crevicular fluid assays as a screening test for periodontal disease. As expected, relationships between total quantity of substances in gingival crevicular fluid and periodontal disease status were unclear. In logistic regression analysis of periodontal disease status, which functioned as the

Table 4. Combinations of significant substances capable of predicting periodontal disease in six regression models

| # of Model | Independent variable #1     | <i>p</i> | Independent variable #2 | <i>p</i> |
|------------|-----------------------------|----------|-------------------------|----------|
| 1          | IgA                         | 0.0131   | Albumin                 | 0.0436   |
| 2          | IgA                         | 0.0098   | Transferrin             | 0.0313   |
| 3          | IgA                         | 0.0272   | Neutrophil elastase     | 0.0196   |
| 4          | Hemoglobin                  | 0.0098   | Neutrophil elastase     | 0.0189   |
| 5          | α <sub>1</sub> -antitrypsin | 0.0394   | Neutrophil elastase     | 0.0486   |
| 6          | IgG                         | 0.0421   | Neutrophil elastase     | 0.0275   |

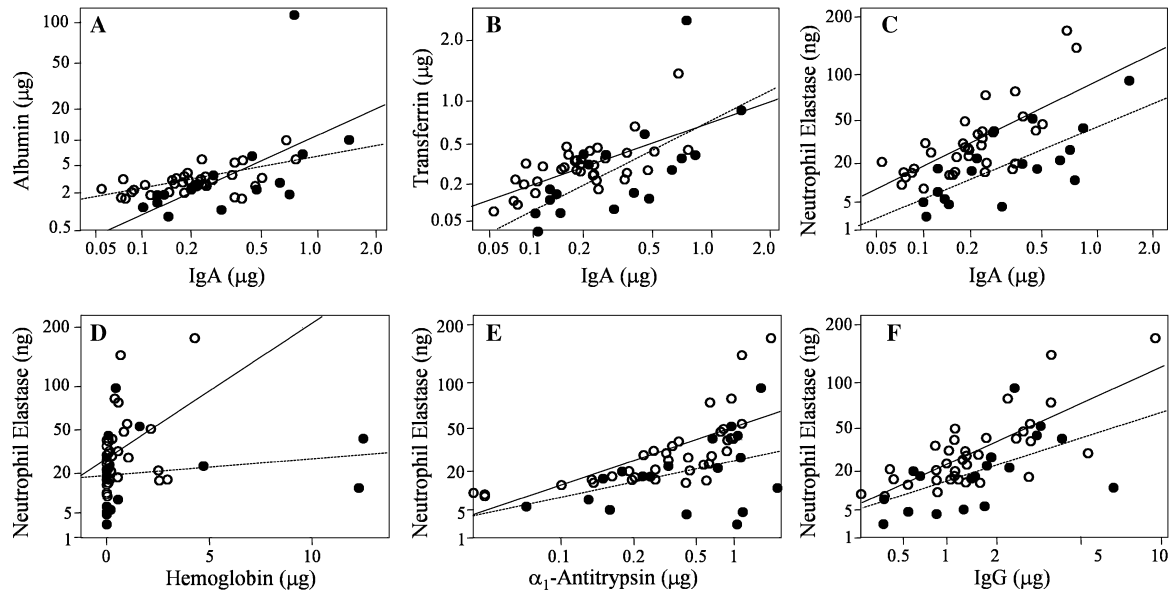


Fig. 1. Scatter plots of total quantities of two substances that were significant in each predictive model. Regression lines were generated for subject with periodontal destruction (SPD: broken line for closed circles) and subject with minimal periodontal destruction (SMD: solid line for open circles) samples.

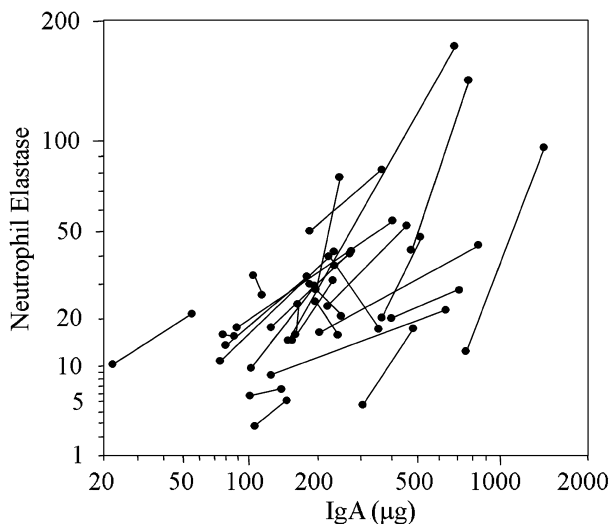


Fig. 2. Scattergram of total amount between neutrophil elastase and IgA: each line is derived from two points, which represent gingival crevicular fluid samples from the same subject.

dependent variable, the output consisted of the combination of total amounts of two substances. Scatter plots constructed from such substances indicated that the combination of IgA and neutrophil elastase was the most satisfactory predictive model of periodontal disease status. Subsequently, IgA and neutrophil elastase were employed in the predictive model of periodontal disease status; additionally,

sensitivity and specificity of the test were satisfactory.

Among 12 substances in gingival crevicular fluid evaluated in logistic regression models, IgA and neutrophil elastase, individually, displayed meaningful predictive ability with respect to SPD. Furthermore, combination of IgA and neutrophil elastase afforded the most predictive model. Thus, IgA and neutrophil elastase may be key

substances for prediction of SPD. Protective roles of IgA in gingival crevicular fluid against periodontal destruction have been demonstrated (16–18). IgA in gingival crevicular fluid was associated with periodontal disease (16). In saliva, secretory IgA constitutes the main specific immune defense mechanism and may play an important role in the homeostasis of oral microorganisms (19). Detection of IgA antibodies specific to *Porphyromonas gingivalis* in gingival crevicular fluid could be utilized as a predictive parameter of periodontal infection (20). Neutrophil elastase, which was derived from the effector system of acute inflammatory response, was also identified in gingival crevicular fluid of patients with periodontal disease (21).

Biological rationale as to the connection between IgA and neutrophil elastase in gingival crevicular fluid is interesting. Predicted value, which was calculated from transformed amounts of IgA and neutrophil elastase, may express degree of relative connection between systems for protection by IgA and for acute inflammatory response. In the present investigation, relative rate of the quantity of IgA transformed to neutrophil elastase in gingival crevicular fluid was higher in SPD than

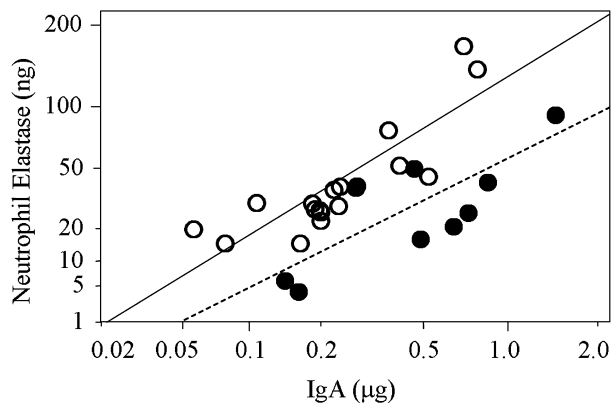


Fig. 3. Scatter plots of total amount with higher values of neutrophil elastase and IgA in two samples from the same subject. Regression lines represent subject with periodontal destruction (SPD: broken line for closed circles) and subject with minimal periodontal destruction (SMD: solid line for open circles).

that in SMD. Thus, an imbalance between both systems might appear in the gingival crevicular fluid of SPD. Recent studies regarding intensive monitoring of periodontal disease status focused on matrix metalloproteinase in gingival crevicular fluid (22–24). In IgA nephropathy, an IgA-specific disease in kidney, matrix metalloproteinases play an important role in mesangial cell activation (25, 26). These findings may underscore the importance of the role of IgA observed in this study.

Procedures capable of predicting periodontal disease status developed in the present investigation may be uncomplicated: two samples of gingival crevicular fluid were obtained via placement of a standardized paper strip at fixed sites for 30 s. Samples, which were stored in sampling tubes,

were then delivered to the commercial laboratory for biochemical assay. The assays were conducted routinely in the laboratory; thus, these protocols could be performed in bulk in a satisfactory manner.

On the scatter plots, the lines between two points, which represented data derived from two sites in the same subject, were nearly parallel to regression lines. The other short lines, which were directed perpendicular to the regression line, indicated that similar quantities were obtained from both sites. These findings revealed that, despite the differing quantities of gingival crevicular fluid occurring in two samples from the same subject, the relative relation between IgA and neutrophil elastase is consistent between the two samples. In other words, the amount of gingival crevicular fluid may exert little

influence on the relative relation. The difference in the relative relation between IgA and neutrophil elastase in gingival crevicular fluid was consistent throughout the range of approximately 100-fold in terms of the amounts in gingival crevicular fluid. This trend may increase the availability of gingival crevicular fluid assays for screening purposes. The quantity of gingival crevicular fluid may be limited; however, the sample could be utilized for prediction of periodontal disease status. Amount of gingival crevicular fluid did not display normal distribution for all substances. Power transformation of the amounts of gingival crevicular fluid may also contribute to these promising results.

Mean values of pocket depth and attachment loss at fixed sites were higher than those of the whole mouth. Generally, maxillary molars and mandibular incisors display a potentially greater susceptibility to periodontal destruction relative to other teeth (27). The fixed sites where gingival crevicular fluid samples were collected could reflect periodontal disease status of the subject. Significant differences between SMD and SPD with respect to pocket depth and attachment loss were also apparent at the fixed sites. In contrast to the distinctions in the clinical indices, no significant difference was observed between SMD and SPD in terms of the amount of any substances in gingival crevicular fluid. However, combination of two substances in gingival crevicular fluid at the fixed sites could be crucial for prediction of

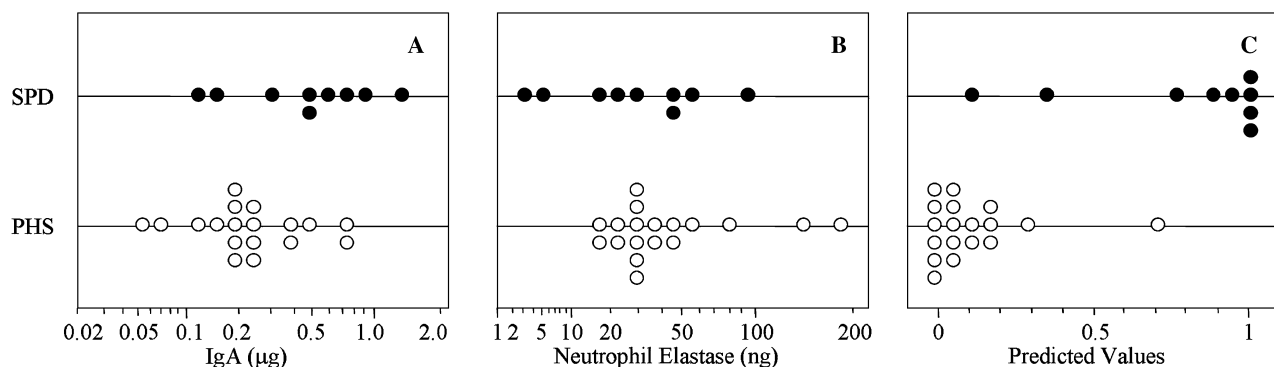


Fig. 4. Comparisons of representative values of IgA (A), neutrophil elastase (B) and predicted values (C) between subject with periodontal destruction (SPD) and subject with minimal periodontal destruction (SMD).

periodontal destruction. Ideally, utilization of a combination of two substances in gingival crevicular fluid collected at any site in the mouth, including healthier sites, may be incorporated for the prediction.

Content of gingival crevicular fluid substances is generally expressed as concentration and/or total amount. In the present study, expression of content in gingival crevicular fluid samples was based on total amount per 30-s sample. In order to express content of gingival crevicular fluid substance as concentration, measurement of gingival crevicular fluid volume is necessary. Gingival crevicular fluid volume in the paper strips that were used for collection of gingival crevicular fluid is determined via electric impedance; thus, the measurement requires a special device as well as an additional procedure. Generally, less expensive, easier screening methods are essential. Several investigators described the superiority of expression as total amount per timed sample (2, 28, 29). Upon comparison of sites characterized by similar amounts of the substance in gingival crevicular fluid, dilution of the substance by fluid may be greater in sites exhibiting larger gingival crevicular fluid volume than in sites with less gingival crevicular fluid volume. In the case of small gingival crevicular fluid volume, which was evident in healthier sites, measurement of fluid volume often resulted in a wide variation of concentration of the substance in gingival crevicular fluid. With respect to the accuracy of measurement, contamination by saliva may exert considerable influence on the concentration of the gingival crevicular fluid substances in sites with less gingival crevicular fluid.

In the present study, combination of IgA and neutrophil elastase in gingival crevicular fluid could afford prediction of periodontal disease status regardless of amount of gingival crevicular fluid. Delivery of samples to the commercial laboratory and biochemical assays were routinely performed in instances involving peripheral blood and urine specimens. In order to establish the gingival crevicular fluid test as a routine method for periodontal disease

screening, additional investigation of more convenient sampling procedures and validation may be necessary.

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