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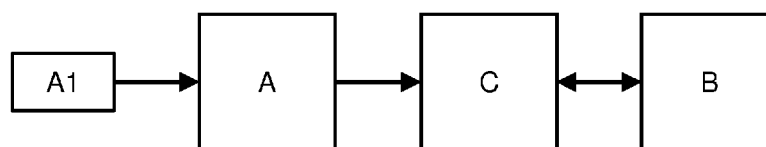


Fig. 1

(57) Abstract: Disclosed is an *in vitro* method for assessing the presence of gingivitis in a human subject. The method is based on the insight to determine a selection of three biomarker proteins. Accordingly, in a saliva sample of the subject the concentrations are measured of the proteins Hepatocyte growth factor (HGF) and Interleukin-1 β (IL-1 β), and at least one of C reactive protein (CRP) and Haemoglobin. Based on the concentrations as measured, a value is determined reflecting the joint concentrations for said proteins. This value is compared with a threshold value reflecting in the same manner the joint concentrations associated with the absence of gingivitis or periodontitis. The comparison allows to assessing whether the testing value is indicative of the presence of gingivitis in said subject. Thereby, typically, a testing value reflecting a joint concentration above the joint concentration reflected by the threshold, is indicative of the presence of gingivitis.



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DIAGNOSTICS OF GINGIVITIS BASED ON SALIVARY IL-1BETA AND HGF

FIELD OF THE INVENTION

The invention is in the field of oral care, and pertains to saliva-based diagnostics of periodontal disease. Particularly, the invention pertains to a kit and method for determining the occurrence of gingivitis.

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BACKGROUND OF THE INVENTION

Gum inflammation, or gingivitis, is a non-destructive periodontal disease caused mainly by the adherence of dental bacterial biofilms, or dental plaque, to the tooth surface. If not detected and treated, the reversible gingivitis usually leads to the inflammation of the tissues surrounding the tooth (i.e. periodontal tissues), a condition defined as periodontitis, which is irreversible and causes tissue destruction and alveolar bone loss, and ultimately results in the loss of teeth. During the progression of gum disease, there are usually clinical signs and symptoms associated with it, such as the swelling of the gums, the change in color from pink to dark red, the bleeding of the gums, bad breath, and the gums becoming more tender or painful to touch.

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Severe periodontal inflammation should prompt investigation of conditions such as diabetes mellitus, HIV infection, thrombocytopenia, and leukaemia. For example, in the case of a diabetic person, severe periodontal disease may be a strong predictor of various diabetic complications, including nephropathy, stroke, transient ischemic attack, angina, myocardial infarction, and heart failure.

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Early and accurate diagnosis of periodontal disease, thus, is important from a health perspective. To this end, diagnosis particularly at a stage of gingivitis is desired.

Periodontal diseases are still poorly diagnosed in general dental practice (Morgan RG (2001). Brit Dent J 191: 436-41), resulting in relatively low rates of therapeutic intervention and significant amounts of untreated cases. Current diagnosis relies on imprecise, subjective clinical examination of oral tissue condition (color, swelling, extent of bleeding on probing, probing pocket depth; and bone loss from oral x-rays) by dental professionals. These conventional methods are time consuming, and some of the techniques used (pocket-depth, x-ray) reflect historic events, such as past disease activity, rather than

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current disease activity or susceptibility to further disease. Hence, more objective, faster, accurate, easier-to-use diagnostics which preferably may also be performed by non-specialists are desirable. Thereby it is desirable to measure current disease activity, and possibly a subject's susceptibility to further periodontal disease.

5 Saliva or oral fluids have long been advocated as a diagnostic fluid for oral and general diseases, and with the advent of miniaturized biosensors, also referred to as lab-on-a-chip, point of care diagnostics for rapid chair-side testing have gained greater scientific and clinical interest (Lee et al., Salivary diagnostics. *Orthod Craniofac Res*, 12 (3) (2009), pp. 206–211).

10 Especially for periodontal disease detection, inflammatory biomarkers associated with tissue inflammation and breakdown may easily end up in saliva due to proximity, suggesting saliva has strong potential for periodontal disease detection. Indeed, this area thus has gained significant interest and encouraging results have been presented, yet no definite test has emerged yet.

15 Previous limitations to the development of point-of-care tests for salivary biomarkers included a lack of technologies that were adaptable to chair-side applications and an inability to analyze multiple biomarkers in individual samples. Also the selection of which multiple biomarkers to include in such a test has not been adequately addressed in the literature nor implemented in practical tests. These challenges are discussed in Ji & Choi,
20 Front Cell Infect Microbiol. 2015; 5: 65.

 Biomarkers represent biological indicators that underpin clinical manifestations, and as such are objective measures by which to diagnose clinical outcomes of periodontal disease. Ultimately, proven biomarkers could be utilized to assess risk for future disease, to identify disease at the very earliest stages, to identify response to initial therapy,
25 and to allow implementation of preventive strategies.

 Current methods to diagnose gingivitis involve estimation of pocket depth, the level of bleeding upon probing and gingival index. This is a labor intensive process that a dentist will not perform on every patient and/or on every visit, and is impossible to perform by a consumer (self-diagnosis).

30 It would be desired to provide a simpler process, and particularly a process that requires only that a small saliva sample is taken from a subject, and possibly by the subject him- or herself. It is desired that such a sample be entered into an *in vitro* diagnostic device, which will allow, based on measurement, a classification of the saliva sample such that it can return an indication of the likelihood that the subject has gingivitis.

SUMMARY OF THE INVENTION

In order to better address the foregoing desires, the invention, in one aspect, concerns an *in vitro* method for assessing the presence of gingivitis in a human subject, comprising detecting, in a saliva sample from said human subject, the concentrations of the proteins: Hepatocyte growth factor (HGF); Interleukin-1 β (IL-1 β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb); determining a testing value reflecting the joint concentrations measured for said proteins; comparing the testing value with a threshold value reflecting in the same manner the joint concentrations associated with the absence of gingivitis or periodontitis, so as to assess whether the testing value is indicative of the presence of gingivitis in said subject.

In another aspect, the invention presents the use of the proteins Hepatocyte growth factor (HGF); Interleukin-1 β (IL-1 β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb) in a saliva sample of a human subject, as biomarkers for assessing the presence of gingivitis in said subject. Typically the assessment is made on the basis of the concentrations of said proteins in said sample.

In a further aspect, the invention resides in a system for diagnosing gingivitis in a human subject, the system comprising:

- detection means able and adapted to detect in a sample of saliva of the human patient the proteins HGF, IL-1 β , and at least one of CRP and Hb; and
- a processor able and adapted to determine from the determined concentrations of said proteins an indication of the subject having gingivitis or not.

The system optionally contains a data connection to a user interface, particularly a graphical user interface, capable of presenting information, preferably also capable of putting in information, the interface being either a part of the system or a remote interface;

Optionally one or more of the foregoing items, particularly the processor, are enabled to function "in the cloud", i.e., not on a fixed machine, but by means of an internet-based application.

In a still further aspect, the invention provides a kit for detecting at least three biomarkers for gingivitis in a sample of saliva of a human patient, said kit comprising one or more, typically three, detection reagents, for detecting HGF, IL-1 β and one of CRP and Hb. Typically, three or more detection reagents are used, each of which binds a different biomarker. In one embodiment, a first detection reagent is capable of binding Hepatocyte

growth factor (HGF), a second detection reagent is capable of binding Interleukin-1 β (IL-1 β), and a third detection reagent is capable of binding one of C-reactive protein (CRP) and Haemoglobin (Hb).

In yet another aspect, the invention provides an *in vitro* method for determining a change in status of gingivitis in a human subject over a time interval from a first time point t_1 to a second time point t_2 , the method comprising detecting, in at least one saliva sample obtained from said subject at t_1 and in at least one saliva sample obtained from said subject at t_2 , the concentrations of the proteins: Hepatocyte growth factor (HGF); Interleukin-1 β (IL-1 β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb), and comparing the concentrations, whereby a difference in any one, two or all three concentrations, reflects a change in status.

In a further aspect, the invention provides a method of diagnosing whether a human patient has gingivitis, comprising detecting in a sample of saliva of the human patient the proteins Hepatocyte growth factor (HGF), Interleukin-1 β (IL-1 β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb), and assessing the presence of gingivitis in the patient on the basis of the concentrations of said proteins in said sample. Optionally, the method of this aspect comprises the further step of treating the gingivitis in the patient.

In yet a further aspect, the invention provides a method of detecting the proteins Hepatocyte growth factor (HGF); Interleukin-1 β (IL-1 β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb) in a human patient suffering from gingivitis, comprising:

(a) obtaining a saliva sample from a human patient; and

(b) detecting whether Hepatocyte growth factor (HGF); Interleukin-1 β (IL-1 β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb) are present in the sample by contacting the saliva sample with one or more detection reagents for binding said proteins and detecting binding between each protein and the one or more detection reagents. Typically, there is a first detection reagent capable of binding HGF, a second detection reagent is capable of binding IL-1 β , and a third detection reagent is capable of binding one of CRP and Hb.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 schematically represents a system for use in the method as described in this disclosure.

DETAILED DESCRIPTION OF THE INVENTION

In a general sense, the invention is based on the judicious insight that as few as three proteins can serve as biomarkers in a saliva sample of a subject, for a sufficiently accurate determination of whether said subject has gingivitis. These proteins are Hepatocyte growth factor (HGF); Interleukin-1 β (IL-1 β), and either or both of C-reactive protein (CRP) and Haemoglobin (Hb).

HGF is a paracrine cellular growth, motility and morphogenic factor. It is secreted by mesenchymal cells and targets and acts primarily upon epithelial cells and endothelial cells, but also acts on haemopoietic progenitor cells. HGF has been shown to have a major role in myogenesis and in wound healing. Its ability to stimulate mitogenesis, cell motility, and matrix invasion gives it a central role in angiogenesis, tumorigenesis, and tissue regeneration. HGF stimulates growth of epithelial cells and prevents regeneration of the connective tissue attachment. HGF is known as a serum marker indicating disease activity in various diseases.

IL-1 β is a member of the interleukin 1 family of cytokines. This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.

C-reactive protein (CRP) is an annular, pentameric protein found in blood plasma, whose levels rise in response to inflammation. It is an acute-phase protein of hepatic origin that increases following interleukin-6 secretion by macrophages and T cells. Its physiological role is to bind to lysophosphatidylcholine expressed on the surface of dead or dying cells (and some types of bacteria) in order to activate the complement system via the C1Q complex. CRP is synthesized by the liver in response to factors released by macrophages and fat cells (adipocytes). It is a member of the pentraxin family of proteins.

Haemoglobin (Hb) is the iron-containing oxygen-transport metalloprotein in the red blood cells of nearly all vertebrates as well as the tissues of some invertebrates.

The four proteins mentioned above are known in the art. The person is aware of their structure, and of methods to detect them in an aqueous sample, such as a saliva sample. Hereinafter the aforementioned protein biomarkers are collectively referred to as “the biomarker panels of the invention.” A biomarker panel of the invention, in one embodiment, consist of the four protein biomarkers identified in the invention, i.e., HGF, IL-1 β , CRP, and

Hb. Preferably, a biomarker panel of the invention consists of not more than three of the protein biomarkers identified in the invention, i.e., HGF, IL-1 β , and CRP or HGF, IL-1 β , and Hb. In addition to the biomarker panels of the invention, other biomarkers and or data, such as demographic data (e.g. age, sex) can be included in a set of data applied for the determination of gingivitis. Preferred additional biomarkers are Interleukin-6 (IL6) and elastase.

When other biomarkers are optionally included, the total number of biomarkers (i.e. the biomarker panel of the invention plus additional biomarkers) is typically 4, 5 or 6.

However, a desirable advantage of the present invention is that the occurrence of gingivitis in a subject can be determined by measuring not more than four biomarkers, and preferably measuring only three biomarkers. Particularly, the determination does not need to involve the use of other data, which advantageously provides a simple and straightforward diagnostic test.

The method, as desired, requires only that a small saliva sample, e.g. a dropsize, is taken from the subject. The size of the sample will typically range from 0.1 μ l to 2 ml, such as 1-2 ml, whereby smaller amounts, e.g., 0.1 to 100 μ l can be used for *in vitro* device processing, and whereby taking a larger sample, such as up to 20 ml, such as 7.5 to 17 ml, is also possible. This sample is entered into an *in vitro* diagnostic device, which measures the concentrations of the at least three proteins involved, and which returns a diagnostic outcome, classifying the subject on the basis of a likelihood of having gingivitis or not.

The ease of use of this invention will make it possible to test the majority of dental patients on a regular basis (e.g. as part of a regular dental check or even at home). This allows detecting the presence of gingivitis before it proceeds to periodontitis, and thus enables taking timely oral care measures to reverse the effects of the gingivitis. Particularly, the method is also suitable for self-diagnosis, whereby the steps of taking the sample and entering it into a device can be conducted by the subject him- or herself.

A method of the invention typically comprises detecting the aforementioned at least three proteins making up a biomarker panel of the invention, and optional further biomarker proteins, by using one or more detection reagents.

The "saliva" that is tested according to the invention may be undiluted saliva, which may be obtained by spitting or swabbing, or diluted saliva, which may be obtained by rinsing the mouth with a fluid. Diluted saliva may be obtained by the patient rinsing or swilling their mouth for a few seconds with sterile water (for example 5ml or 10ml) or other

suitable fluid, and spitting into a container. Diluted saliva may sometimes be referred to as an oral rinse fluid.

By “detecting” is meant measuring, quantifying, scoring, or assaying the concentration of the biomarker proteins. Methods of evaluating biological compounds, including biomarker proteins, are known in the art. It is recognized that methods of detecting a protein biomarker include direct measurements and indirect measurements. One skilled in the art will be able to select an appropriate method of assaying a particular biomarker protein.

The term “concentration” with respect to the protein biomarkers is to be given its usual meaning, namely the abundance of the protein in a volume. Protein concentration is typically measured in mass per volume, most typically mg/ml or µg/ml, but sometimes as low as pg/ml. An alternative measure is Molarity (or Molar concentration), mol/L or “M”. The concentration can be determined by detecting the amount of protein in a sample of known, determined or pre-determined volume.

An alternative to determining the concentration is to determine the absolute amount of the protein biomarker in the sample, or determining the mass-fraction of the biomarker in the sample, for example the amount of the biomarker relative to the total of all other proteins in the sample.

A “detection reagent” is an agent or compound that specifically (or selectively) binds to, interacts with or detects the protein biomarker of interest. Such detection reagents may include, but are not limited to, an antibody, polyclonal antibody, or monoclonal antibody that preferentially binds the protein biomarker.

The phrase “specifically (or selectively) binds” or “specifically (or selectively) immunoreactive with,” when referring to a detection reagent, refers to a binding reaction that is determinative of the presence of the protein biomarker in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified detection reagent (e.g. antibody) binds to a particular protein at least two times the background and does not substantially bind in a significant amount to other proteins present in the sample. Specific binding under such conditions may require an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays (enzyme-linked immunosorbent assay) are routinely used to select antibodies specifically immunoreactive with a protein (*see, e.g.*, Harlow & Lane, *Antibodies, A Laboratory Manual* (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity). Typically a specific or

selective reaction will be at least twice the background signal or noise and more typically more than 10 to 100 times the background.

"Antibody" refers to a polypeptide ligand substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, which specifically binds and recognizes an epitope (e.g., an antigen). The recognized immunoglobulin genes include the kappa and lambda light chain constant region genes, the alpha, gamma, delta, epsilon and mu heavy chain constant region genes, and the myriad immunoglobulin variable region genes. Antibodies exist, e.g., as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. This includes, e.g., Fab' and F(ab)'₂ fragments. The term "antibody," as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized de novo using recombinant DNA methodologies. It also includes polyclonal antibodies, monoclonal antibodies, chimeric antibodies, humanized antibodies, or single chain antibodies. "Fc" portion of an antibody refers to that portion of an immunoglobulin heavy chain that comprises one or more heavy chain constant region domains, CH₁, CH₂ and CH₃, but does not include the heavy chain variable region. The antibody may be a bispecific antibody, e.g. an antibody that has a first variable region that specifically binds to a first antigen and a second variable region that specifically binds to a second, different, antigen. Use of at least one bispecific antibody can reduce the number of detection reagents needed.

Diagnostic methods differ in their sensitivity and specificity. The "sensitivity" of a diagnostic assay is the percentage of diseased individuals who test positive (percent of "true positives"). Diseased individuals not detected by the assay are "false negatives." Subjects who are not diseased and who test negative in the assay, are termed "true negatives." The "specificity" of a diagnostic assay is 1 minus the false positive rate, where the "false positive" rate is defined as the proportion of those without the disease who test positive.

The biomarker protein(s) of the invention can be detected in a sample by any means. Preferred methods for biomarker detection are antibody-based assays, protein array assays, mass spectrometry (MS) based assays, and (near) infrared spectroscopy based assays. For example, immunoassays, include but are not limited to competitive and non-competitive assay systems using techniques such as Western blots, radioimmunoassays, ELISA, "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, fluorescent immunoassays and the like. Such assays are routine and well known in the art. Exemplary immunoassays are described briefly below (but are not intended by way of limitation).

Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis buffer such as RIPA buffer (1% NP-40 or Triton X-100, 1 % sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1 % Trasyolol) supplemented with protein phosphatase and/or protease inhibitors (e.g., EDTA, PMSF, aprotinin, sodium vanadate), adding an antibody of interest to the cell lysate, incubating for a period of time (e.g., 1-4 hours) at 4°C, adding protein A and/or protein G sepharose beads to the cell lysate, incubating for about an hour or more at 4°C, washing the beads in lysis buffer and re-suspending the beads in SDS/sample buffer. The ability of the antibody to immunoprecipitate a particular antigen can be assessed by, e.g., western blot analysis. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the binding of the antibody to an antigen and decrease the background (e.g., pre-clearing the cell lysate with Sepharose beads).

Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel (e.g., 8%-20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, blocking the membrane in blocking solution (e.g., PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer (e.g., PBS-Tween 20), blocking the membrane with primary antibody (the antibody of interest) diluted in blocking buffer, washing the membrane in washing buffer, blocking the membrane with a secondary antibody (which recognizes the primary antibody, e.g., an anti-human antibody) conjugated to an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) or radioactive molecule (e.g., ³²P or ¹²⁵I) diluted in blocking buffer, washing the membrane in wash buffer, and detecting the presence of the antigen. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected and to reduce the background noise.

ELISAs typically comprise preparing antigen (i.e. the biomarker protein of interest or fragment thereof), coating the well of a 96- well microtiter plate with the antigen, adding the antibody of interest conjugated to a detectable compound such as an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antigen. In ELISAs the antibody of interest does not have to be conjugated to a detectable compound; instead, a second antibody (which recognizes the antibody of interest) conjugated to a detectable compound may be added to the well. Further, instead of coating the well with the antigen, the antibody may be coated to the well. In this case, a second antibody conjugated to a detectable compound may be added

following the addition of the antigen of interest to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art.

In the methods disclosed herein, the concentration of the aforementioned at least three biomarker proteins is determined. It was found that this represents a selection of biomarker proteins that allows providing both sensitivity and specificity exceeding 70%, and preferably exceeding 75%, in the determination of the presence of gingivitis. Such a test is considered to satisfy the requirements for a test that is feasible in the field (particularly: adequately predictable, yet as simple as possible).

Since multiple markers are used, not all of the biomarkers need to exhibit a significant differential expression in order for the presence of gingivitis to be determined. In an embodiment, in which the threshold is a value reflecting a healthy condition, an appreciable change, such as an increase, in the concentration of at least two of the biomarkers (i.e., differential expression) indicates that the subject is likely to have gingivitis. Preferably an appreciable change in the concentration of two or all three biomarkers indicates that the subject has gingivitis. The change may be an increase for all biomarkers, but also may be, e.g., a decrease of one of the biomarkers and an increase of one or two of the other biomarkers. The insight reflected in the present disclosure is that gingivitis can be distinguished from a healthy oral condition with sufficient accuracy based on a measurement of a biomarker panel of the invention.

This insight supports another aspect, the invention, which is the use of the proteins Hepatocyte growth factor (HGF); Interleukin-1 β (IL-1 β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb), as biomarkers in a saliva sample of a human subject, for assessing the presence of gingivitis in said subject, typically on the basis of the concentrations of said proteins in said sample.

This use can be implemented in a method as substantially described hereinbefore and hereinafter.

The method of the invention comprises determining a testing value reflecting the joint concentrations measured for said proteins. A joint concentration value can be any value obtained by input of the concentrations as determined and an arithmetic operation of these values. This can, e.g., be a simple addition of the concentrations. It can also involve multiplying each concentration with a factor reflecting a desired weight of these concentrations, and then adding up the results. It can also involve multiplying the concentrations with each other, or any combination of multiplication, division, subtraction,

exponentiation, and addition. It can further involve raising those concentrations to some power.

The resulting joint concentration value is compared with a threshold value reflecting in the same manner the joint concentrations associated with the absence of gingivitis or periodontitis. This threshold value is typically deemed as healthy in terms of gingival health, because it is a value that is not associated with gingivitis or periodontitis. The comparison allows assessing whether the testing value is indicative of the presence of gingivitis in said subject.

The threshold value can, e.g., be the joint concentration value, obtained in the same manner on the basis of the concentrations determined for the same proteins in a reference sample associated with the absence of gingivitis or periodontitis, i.e. in a subject classified as healthy in terms of gingival health. Typically, thereby a testing value reflecting a joint concentration above the joint concentration reflected by the threshold, is indicative of the presence of gingivitis in said subject. However, it will be understood that it is also possible to calculate a threshold value (e.g. by using a negative multiplier) such that a testing value indicating gingivitis would be below the threshold, and a testing value indicating no gingivitis or periodontitis, would be above the threshold.

The threshold value can also be determined on the basis of measuring the concentrations of the present biomarker proteins in a set of samples, including healthy subjects and subjects with a known diagnosis of gingivitis. Thereby the measured concentration values can be subjected to statistical analysis, possibly including machine learning methods, allowing to discriminate, with the desired sensitivity and specificity, subjects classified as healthy and subjects classified as patients suffering from gingivitis. Therefrom, the desired threshold value can be obtained. On the basis of this threshold value, a sample to be tested can be subjected to the same concentration measurement, and the concentration values are then processed, in the same manner in which the threshold value is obtained, so as to determine a joint concentration value that can be compared with the threshold, thus allowing the tested sample to be classified as being healthy or being indicative of the presence of gingivitis.

In an interesting embodiment, the joint concentration value is obtained in the form of a score as follows. A numerical value (protein concentration values in e.g. ng/ml) is assigned to each measurement, and these values are used in a linear or non-linear combination to calculate a score between zero and one. In the event that the threshold value is determined on the basis of a set of subjects as mentioned above, the score between 0 and 1

is typically calculated with the sigmoid function that takes the joint concentration as input (as shown further on).

When the score exceeds a certain threshold, the method indicates that the patient has gingivitis. The threshold may be chosen based on the desired sensitivity and specificity. In another interesting embodiment the same score is calculated between zero and one but now reflects the likelihood of having gingivitis (such that a professional can interpret this in view of his/her total assessment of the patient). Alternatively the score is converted to a qualified indication stating e.g. low, medium or high risk of having gingivitis.

It will be understood that in performing a 'health or gingivitis classification' on a subject, in accordance with the invention, this is on subjects that can be assumed not to suffer from more severe oral health inflammatory conditions, such as periodontitis. This can either be known from e.g. a previously performed diagnosis of periodontitis, or, e.g., assumed from the subject's oral health condition record.

Clinical definitions as acknowledged in the art, and as applied in this disclosure, are based on the following:

Gingival Index (GI)

A full mouth gingival index will be recorded based on the Lobene Modified Gingival Index (MGI) rated on a scale of 0 to 4, where:

- 0 = absence of inflammation,
- 1 = mild inflammation; slight change in color little change in texture of any portion of but not the entire margin or papillary gingival unit,
- 2 = mild inflammation; but involving entire margin or papillary unit,
- 3 = moderate inflammation; glazing, redness, oedema and/or hypertrophy of margin or papillary unit,
- 4 = severe inflammation; marked redness, oedema and/or hypertrophy of marginal or papillary gingival unit, spontaneous bleeding, congestion, or ulceration].

Probing depths (PD)

Probing depths will be recorded to the nearest mm using a manual UNC-15 periodontal probe. Probing depth is the distance from the probe tip (assumed to be at the base of the pocket) to the free gingival margin.

Gingival recession (REC)

Gingival recession will be recorded to the nearest mm using a manual UNC-15 periodontal probe. Gingival recession is the distance from the free gingival margin to the

cemento-enamel junction. Gingival recession will be indicated as a positive number and gingival overgrowth will be indicated as a negative number.

Clinical attachment loss (CAL)

Clinical attachment loss will be calculated as the sum of probing depth +
5 recession at each site

Bleeding on probing (BOP)

Following probing, each site will be assessed for bleeding on probing, if
bleeding occurs within 30s of probing, a score of 1 will be assigned for the site, otherwise a
score of 0 will be assigned.

10 The resulting subject group (patient group) definition is as follows:

- Healthy group (H): $PD \leq 3$ mm in all sites (but would allow up to four 4 mm
pockets at distal of last standing molars), no sites with interproximal attachment loss, GI of $\geq$
2.0 in $\leq 10\%$ sites, %BOP scores $\leq 10\%$;
- Gingivitis group (G): GI ≥ 3.0 in $> 30\%$ of sites, no sites with interproximal
15 attachment loss, no sites with $PD > 4$ mm, %BOP scores $> 10\%$;
- Mild-moderate periodontitis group (MP): interproximal PD of 5-7 mm,
(equating to approximately 2-4 mm CAL) at ≥ 8 teeth, %BOP scores $> 30\%$
- Advanced periodontitis group (AP): interproximal PD of ≥ 7 mm, (equating to
approximately ≥ 5 mm CAL) at ≥ 12 teeth, %BOP scores $> 30\%$

20 In an embodiment, the method of the invention makes use of a system as
represented schematically in Fig. 1. The system can be a single apparatus having various
device components (units) integrated therein. The system can also have its various
components, or some of these components, as separate apparatuses. The components shown
in Fig. 1 are a measurement device (A), a graphical user interface (B) and a computer
25 processing unit (C).

As mentioned above, the system of the invention comprises a data connection
to an interface, whereby the interface itself can be a part of the system or can be a remote
interface. The latter refers to the possibility to use a different apparatus, preferably a
handheld apparatus such as a smartphone or a tablet computer, for providing the actual
30 interface. The data connection in such cases will preferably involve wireless data transfer
such as by Wi-Fi or Bluetooth, or by other techniques or standards.

The measurement device (A) is configured to receive a saliva sample, for
example by putting a drop of saliva on a cartridge (A1), which can be inserted into the device
(A). The device can be an existing device that is capable to determine, from the same saliva

sample, the concentrations of at least the proteins HGF and IL-1 β , and at least one of the proteins CRP and Hb. The processing unit (C) receives numerical values for the protein concentrations from part (A). The unit (C) is provided with software (typically embedded software) allowing it to calculate a score (S) between 0 and 1. The software further includes a numerical value for a threshold (T). If the calculated value (S) exceeds (T), unit (C) will output an indication (I) of 'gingivitis' to the graphical user interface (B), otherwise it will output 'no gingivitis'. A further embodiment may use the specific value of (S) to indicate the certainty with which the indication (I) is made. This can be a probability score, whereby 0.5 is a possible threshold value, and e.g. a score S=0.8 would indicate the probability of gingivitis. Interesting options are:

- Based on the score S, one can directly indicate a certainty, i.e. S=0.8 means 80% certainty of gingivitis;
- Based on the score S one can make a binary or tertiary indication:
- S<T -> healthy, S $\geq$ T -> gingivitis
- S<R1 -> healthy, R1 $\leq$ S<R2 -> inconclusive,
- S $\geq$ R2 -> gingivitis

In addition, it is possible to attach a certainty to such a binary or tertiary indication. This certainty will be determined by the distance of the score S from the chosen threshold(s) (T,R1,R2).

* A specific calculation of the score can be implemented, e.g. by means of a sigmoid function, applying the following formula:

$$S = \frac{1}{1 + \exp(-(c_0 + \sum_{i=1}^N c_i B_i))}$$

Therein N is the number of proteins/biomarkers used. c_0 , c_1 , etc. are coefficients (numerical values) and B_1 , B_2 , etc. are the respective protein concentrations.

* Determining of the coefficients can be done by a training procedure:

- Select N1 subjects with gingivitis, and N2 subjects without gingivitis. The subjects without gingivitis are considered to have score S=0, the subjects with gingivitis are considered to have score S=1.
- Take a saliva sample from each subject and determine the protein concentrations of a combination of biomarkers as explained above.

- Perform logistic regression between the protein concentrations and the scores.

* Other regression or machine learning methods (linear regression, neural network, support vector machine) may be used to train a classifier that predicts whether a subject has gingivitis or a healthy oral condition based on the protein concentrations.

With reference to the aforementioned system, the invention also provides, in a further aspect, a system for diagnosing gingivitis in a human subject, the system comprising:

- detection means able and adapted to detect in a sample of saliva of a human patient the proteins HGF, IL-1 β , and at least one of CRP and Hb; As explained above, such means are known, and easily accessible to the skilled person. Typically, there is provided a container for receiving an oral sample of a subject therein, the container provided with the detection means;

-

- a processor able and adapted to determine from the determined concentrations of said proteins an indication of the subject having gingivitis or not.

Optionally, the system comprises a user interface (or a data connection to remote interface) particularly a graphical user interface (GUI), capable of presenting information; a GUI is a type of user interface that allows users to interact with electronic devices through graphical icons and visual indicators such as secondary notation, instead of text-based user interfaces, typed command labels or text navigation (none of such interface types being excluded in the present invention); GUIs are generally known, and are used typically in handheld mobile devices such as MP3 players, portable media players, gaming devices, smartphones and smaller household, office and industrial controls; as said, the interface optionally can also be chosen so as to be capable of putting in information, such as, e.g., the age of the subject, sex, BMI (Body Mass Index).

The invention also provides, either separately or as part of the aforementioned system, a kit for detecting at least three biomarkers for gingivitis in a sample of saliva of a human patient. The kit of the invention comprises one or more detection reagents for detecting HGF, IL-1 β and one of CRP and Hb. Typically, the kit comprises three detection reagents, each reagent directed to a different biomarker, wherein a first detection reagent is capable of binding Hepatocyte growth factor (HGF), a second detection reagent is capable of binding Interleukin-1 β (IL-1 β), and a third detection reagent is capable of binding one of C-reactive protein (CRP) and Haemoglobin (Hb). As discussed above with reference to the method of the invention, the kit may comprise more detection reagents, such as particularly

for the other of CRP and Hb, and/or for IL6 and/or elastase. In a preferred embodiment the detection reagents made available in the kit consist of the detection reagents for the selection of three proteins making up a 3-biomarker panel of the invention, as mentioned.

Preferably said kits comprise a solid support, such as a chip, a microtiter plate or a bead or resin comprising said detection reagents. In some embodiments, the kits comprise mass spectrometry probes, such as ProteinChipTM.

The kits may also provide washing solutions and/or detection reagents specific for either unbound detection reagent or for said biomarkers (sandwich type assay).

In an interesting aspect, the recognition of a biomarker panel of the invention is applied in monitoring the status of gingivitis in a subject, particularly a human subject, over time. Accordingly, the invention also provides an *in vitro* method for determining a change in status of gingivitis in a human subject over a time interval from a first time point t_1 to a second time point t_2 . The method comprises detecting, in at least one saliva sample obtained from said subject at t_1 and in at least one saliva sample obtained from said subject at t_2 , the concentrations of the proteins: Hepatocyte growth factor (HGF); Interleukin-1 β (IL-1 β), and at least one C-reactive protein (CRP) and Haemoglobin (Hb). The concentrations obtained in both measurements are compared, whereby a difference of preferably at least two, and more preferably all three concentrations, reflects a change in status. This difference can be reviewed as a difference in concentrations, thus allowing a direct comparison without first generating a number between 0 and 1, or any other classification. It will be understood that the measurements received at both points in time can also be processed in just the same manner as done when determining the absence or occurrence of gingivitis as above.

The invention also provides a method of diagnosing whether a human patient has gingivitis, comprising detecting in the patient's saliva the presence of the proteins Hepatocyte growth factor (HGF), Interleukin-1 β (IL-1 β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb), and assessing the presence of gingivitis in the patient on the basis of the concentrations of said proteins in said sample. Optionally, the method of this aspect comprises the further step of treating the gingivitis in the patient. This optional treatment step can comprise the administration of known therapeutic agents or dental procedures, or a combination of therapeutic agents and dental procedures. Known therapeutic agents include the administration of antimicrobial-containing agents such as a mouthwash, chip, gel or microsphere. A typical antimicrobial agent for use in treating gingivitis is chlorhexidine. Other therapeutic agents include antibiotics, typically orally-

administered antibiotics, and enzyme suppressants such as doxycycline. Known non-surgical therapeutic procedures include scaling, or scaling and root planing (SRP).

The invention further provides a method of detecting the proteins Hepatocyte growth factor (HGF); Interleukin-1 β (IL-1 β), and at least one of C-reactive protein (CRP)

5 and Haemoglobin (Hb) in a patient suffering from gingivitis, comprising:

(a) obtaining a saliva sample from a human patient; and

(b) detecting whether Hepatocyte growth factor (HGF); Interleukin-1 β (IL-1 β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb) are present in the saliva sample by contacting the saliva sample with one or more detection reagents capable of
10 binding said proteins and detecting binding between each protein and the one or more detection reagents.

The invention will be further illustrated with reference to the following non-
15 limiting example.

Example

In a clinical study with 54 subjects, 25 of whom were diagnosed with gingivitis and 29 had healthy gums, ROC (Receiver-Operator-Characteristic) Area-Under-the
20 Curve (AUC) values were obtained.

In statistics, a receiver operating characteristic curve, or ROC curve, is a graphical plot that illustrates the performance of a binary classifier system as its discrimination threshold is varied. The curve is created by plotting the true positive rate (TPR) against the false positive rate (FPR) at various threshold settings. The true-positive rate is also known as sensitivity, recall or *probability of detection*^[1] in machine learning. The false-positive rate is also known as the fall-out or *probability of false alarm*^[1] and can be calculated as (1 – specificity). The ROC curve is thus the sensitivity as a function of fall-out. In general, if the probability distributions for both detection and false alarm are known, the ROC curve can be generated by plotting the cumulative distribution function (area under the
30 probability distribution from $-\infty$ to the discrimination threshold) of the detection probability on the y-axis, versus the cumulative distribution function of the false-alarm probability on the x-axis. The accuracy of the test depends on how well the test separates the group being tested into those with and without the disease in question. Accuracy is measured by the area under the ROC curve. An area of 1 represents a perfect test; an area of 0.5 represents a worthless

test. A guide for classifying the accuracy of a diagnostic test is the traditional academic point system:

- 0.90-1 = excellent (A)
- 0.80-0.90 = good (B)
- 5 - 0.70-0.80 = fair (C)
- 0.60-0.70 = poor (D)
- 0.50-0.60 = fail (F)

Based on the foregoing, in the results of the aforementioned clinical study, an ROC AUC value of above 0.75 is considered to represent a desirable accuracy for providing a diagnostic test in accordance with the invention.

Table 1 below summarizes, from among all biomarkers and biomarker panels determined, the data representing an ROC AUC value of above 0.70.

The biomarker proteins presented in the table are HGF, IL-1 β , CRP, and Hb as mentioned above, as well as Interleukin-6 (L6) and elastase. In the table, it can be seen that maximum results, in terms of ROC AUC of 0.81 are obtained with biomarker panel consisting of four markers:

HGF, IL-1 β , CRP, and elastase. A result close to this, at 0.80, is obtained with panels of five biomarkers, viz. HGF, IL-1 β , CRP, Hb, and elastase or HGF, IL-1 β , CRP, IL6 and elastase, or even a 6-marker panel.

However, for the purpose of providing a simple, yet adequately reliable test useful in the present invention, it is desired to apply as few biomarkers as possible. It can thereby be seen that the biomarker panels of 1 marker and 2 markers provide ROC AUC values of at or below 0.75, i.e., closer to the aforementioned value of a worthless test (0.5) than to a perfect test (1).

It has now been found that the optimal results, of having a ROC AUC above 0.75 applying as few markers as possible, is contingent on the selection of a biomarker panel consisting of HGF and IL-1 β and either of CRP and Hb (3-marker panel) or both CRP and Hb (4-marker panel). Clearly, with the results being similar, the 3-marker panels are preferred over the 4-marker panel.

Table 1

HGF	IL-1 β	CRP	Haemoglobin	IL6	Elastase	ROC AUC
<i>1 marker</i>						
X						0.73
<i>2 markers</i>						
X	X					0.74
X		X				0.71
X			X			0.75
<i>3 markers</i>						
X	X	X				0.78
X	X		X			0.77
<i>4 markers</i>						
X	X	X	X			0.77
X	X	X			X	0.81
X	X		X		X	0.78
X	X		X	X		0.77
<i>5 markers</i>						
X	X	X	X		X	0.80
X	X	X		X	X	0.80
X	X		X	X	X	0.78
<i>6 markers</i>						
X	X	X	X	X	X	0.80

While the invention has been illustrated and described in detail in the drawings and foregoing description, such illustration and description are to be considered illustrative or exemplary and not restrictive; the invention is not limited to the disclosed embodiments.

For example, it is possible to present detection reagents for different biomarkers in different units. Or, conveniently, a kit of the invention can comprise a fixed set of detection reagents for the protein biomarkers that are used in all embodiments, i.e., HGF and IL-1 β , and flexible modules comprising a detection reagent for either of the further biomarkers, i.e., CRP or Hb.

Other variations to the disclosed embodiments can be understood and effected by those skilled in the art in practicing the claimed invention, from a study of the drawings, the disclosure, and the appended claims. In the claims, the word "comprising" does not exclude other elements or steps, and the indefinite article "a" or "an" does not exclude a plurality. The mere fact that certain features of the invention are recited in mutually different dependent claims does not indicate that a combination of these features cannot be used to advantage. Any reference signs in the claims should not be construed as limiting the scope.

In sum, we hereby disclose an *in vitro* method for assessing the presence of gingivitis in a human subject. The method is based on the insight to determine a selection of three biomarker proteins. Accordingly, in a saliva sample of the subject the concentrations are measured of the proteins Hepatocyte growth factor (HGF) and Interleukin-1 β (IL-1 β), and at least one of C reactive protein (CRP) and Haemoglobin. Based on the concentrations as measured, a value is determined reflecting the joint concentrations for said proteins. This value is compared with a threshold value reflecting in the same manner the joint concentrations associated with the absence of gingivitis or periodontitis. The comparison allows to assessing whether the testing value is indicative of the presence of gingivitis in said subject. Thereby, typically, a testing value reflecting a joint concentration above the joint concentration reflected by the threshold, is indicative of the presence of gingivitis.

CLAIMS:

1. An *in vitro* method for assessing the presence of gingivitis in a human subject, comprising detecting, in a saliva sample from said human subject, the concentrations of the proteins: Hepatocyte growth factor (HGF); Interleukin-1 β (IL-1 β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb); determining a testing value reflecting the joint concentrations measured for said proteins; comparing the testing value with a threshold value reflecting in the same manner the joint concentrations associated with the absence of gingivitis or periodontitis, so as to assess whether the testing value is indicative of the presence of gingivitis in said subject.
2. A method according to claim 1, wherein the threshold value is based on the concentrations determined for the proteins in one or more reference samples, each sample associated with the absence of gingivitis or periodontitis.
3. A method according to claim 1, wherein the threshold value is based on the concentrations of the proteins in a set of samples, including samples from subjects that have no gingivitis or periodontitis and samples from subjects having gingivitis.
4. A method according to any one of the preceding claims, wherein the proteins detected consist of HGF, IL-1 β , and CRP.
5. A method according to any one of the claims 1-3, wherein the proteins detected consist of HGF, IL-1 β , and Hb.
6. A method according to any one of the preceding claims, wherein the concentration values determined are arithmetically processed into a number between 0 and 1.
7. The use of the proteins Hepatocyte growth factor (HGF); Interleukin-1 β (IL-1 β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb) in a saliva sample of a human subject, as biomarkers for assessing the presence of gingivitis in said subject.

8. A kit for detecting at least three biomarkers for gingivitis in a sample of saliva of a human patient, said kit comprising one or more detection reagents for detecting Hepatocyte growth factor (HGF), Interleukin-1 β (IL-1 β), and one of C-reactive protein (CRP) and Haemoglobin (Hb).
9. A kit according to claim 8, wherein the one more detection reagents comprise at least three detection reagents, a first detection reagent for detecting Hepatocyte growth factor (HGF), a second detection reagent for detecting Interleukin-1 β (IL-1 β), and a third detection reagent for detecting one of C-reactive protein (CRP) and Haemoglobin (Hb).
10. A kit according to claim 8 or claim 9, wherein the one or more detection reagents are contained on a solid support.
11. A kit according to any of claims 8 to 10, wherein the one or more detection reagents consist of detection reagents for HGF, IL-1 β , and CRP, or HGF, IL-1 β , and Hb.
12. A system for diagnosing gingivitis in a human subject, the system comprising:
- detection means able and adapted to detect in a sample of saliva of the human patient the proteins HGF, IL-1 β , and at least one of CRP and Hb;
 - a processor able and adapted to determine from the determined concentrations of said proteins an indication of the subject having gingivitis or not.
13. A system according to claim 12, further comprising a container for receiving an oral fluid sample, the container comprising the detection means.
14. A system according to claim 12 or 13, further comprising:
- a user interface for presenting the indication to a user; and
 - a data connection between the processor and the user interface for transferring the indication from the processor to the user interface.
15. A system according to any of claims 12 to 14, wherein the processor is enabled to function by means of an internet-based application.

16. An *in vitro* method for determining a change in status of gingivitis in a human subject over a time interval from a first time point t_1 to a second time point t_2 , the method comprising detecting, in at least one saliva sample obtained from said subject at t_1 and in at least one saliva sample obtained from said subject at t_2 , the concentrations of the proteins:

- 5 Hepatocyte growth factor (HGF); Interleukin- 1β (IL- 1β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb), and comparing the concentrations, whereby a difference in any one, two or all three of the concentrations, reflects a change in status.

17. A method of diagnosing whether a human patient has gingivitis, comprising
10 detecting in a sample of saliva of the human patient the proteins Hepatocyte growth factor (HGF), Interleukin- 1β (IL- 1β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb), and assessing the presence of gingivitis in the patient on the basis of the concentrations of said proteins in said sample.

15 18. A method of detecting the proteins Hepatocyte growth factor (HGF); Interleukin- 1β (IL- 1β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb) in a human patient suffering from gingivitis, comprising:

(a) obtaining a saliva sample from a human patient; and

(b) detecting whether Hepatocyte growth factor (HGF); Interleukin- 1β
20 (IL- 1β), and at least one of C-reactive protein (CRP) and Haemoglobin (Hb) are present in the sample by contacting the sample with one or more detection reagents capable of binding said proteins and detecting binding between each protein and the one or more detection reagents.

1/1

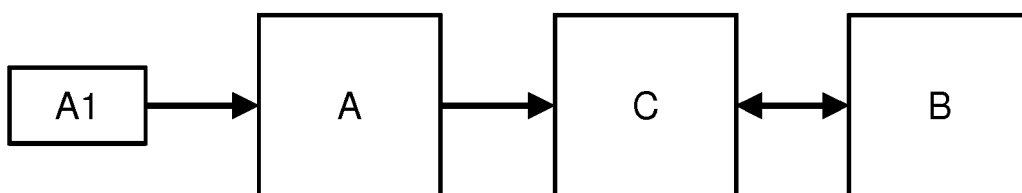


Fig. 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/063658

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68 G01N33/72
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	F.A SCANNAPIECO ET AL: "Salivary Biomarkers Associated with Alveolar Bone Loss", ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, vol. 1098, no. 1, 1 March 2007 (2007-03-01), pages 496-497, XP055102510, ISSN: 0077-8923, DOI: 10.1196/annals.1384.034 abstract; the whole document ----- -/-	1-4,6-18



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 July 2018

Date of mailing of the international search report

11/09/2018

Name and mailing address of the ISA/

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Authorized officer

Schulich, Juliane

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/063658

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KATRIN M. JAEDICKE ET AL: "Salivary cytokines as biomarkers of periodontal diseases", PERIODONTOLOGY 2000, vol. 70, no. 1, 1 February 2016 (2016-02-01), pages 164-183, XP055402461, DK ISSN: 0906-6713, DOI: 10.1111/prd.12117 p. 179	1-4,6-18
X	----- KATRIN M JAEDICKE ET AL: "Validation and quality control of ELISAs for the use with human saliva samples", JOURNAL OF IMMUNOLOGICAL METHODS, ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM, NL, vol. 377, no. 1, 19 January 2012 (2012-01-19), pages 62-65, XP028402826, ISSN: 0022-1759, DOI: 10.1016/J.JIM.2012.01.010 [retrieved on 2012-01-28] p. 63, tables 1 and 2	8-11,18
Y	----- SUK JI ET AL: "Point-of-care diagnosis of periodontitis using saliva: technically feasible but still a challenge", FRONTIERS IN CELLULAR AND INFECTION MICROBIOLOGY, vol. 5, 3 September 2015 (2015-09-03), XP055403123, DOI: 10.3389/fcimb.2015.00065 p. 2-3; p. 5-6; p. 8	1-4,6,7, 12-17
A	----- N. CHRISTODOULIDES ET AL: "Lab-on-a-Chip Methods for Point-of-Care Measurements of Salivary Biomarkers of Periodontitis", ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, vol. 1098, no. 1, 1 March 2007 (2007-03-01), pages 411-428, XP055400313, US ISSN: 0077-8923, DOI: 10.1196/annals.1384.035 the whole document ----- -/--	1-4,6-18

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/063658

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MITSUHIRO OHSHIMA ET AL: "Hepatocyte growth factor (HGF) in jreiodontal disease: detection of HGF in gingival crevicular fluid", JOURNAL OF PERIODONTAL RESEARCH, vol. 37, no. 1, 1 January 2002 (2002-01-01), pages 8-14, XP055491101, ISSN: 0022-3484, DOI: 10.1034/j.1600-0765.2002.00660.x table 1, fig. 2, table 2, p. 9-12 -----	1-4,6,7, 12-17
A	MITSUHIRO OHSHIMA ET AL: "Hepatocyte growth factor in saliva: A possible marker for periodontal disease status.", JOURNAL OF ORAL SCIENCE, vol. 44, no. 1, 1 January 2002 (2002-01-01), pages 35-39, XP055491117, JP ISSN: 1343-4934, DOI: 10.2334/josnurd.44.35 -----	1-4,6-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2018/063658

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

4(completely); 1-3, 6-18(partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 4(completely); 1-3, 6-18(partially)

Assessing gingivitis by using the combination of the following 3 markers: Hepatocyte growth factor (HGF), Interleukin-1beta (IL 1b), C-reactive protein (CRP)

2. claims: 5(completely); 1-3, 6-18(partially)

Assessing gingivitis by using the combination of at the following 3 markers: Hepatocyte growth factor (HGF), Interleukin-1 beta (IL 1 b), Haemoglobin (Hb)
