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(54) Title: A SIMPLE, NON-INVASIVE METHOD FOR DIAGNOSING ORAL CANCER

(57) Abstract: An *in vitro*, non-invasive method is provided for the diagnosis of head and neck cancer. The method comprises quantifying components with a molecular weight lower than a predetermined threshold in a saliva sample obtained from a subject and comparing the so obtained quantification value to a reference value, wherein said predetermined threshold value is obtained by determining a characteristic molecular weight above which the amount of components in the saliva of individuals having tissue alterations associated with a head and neck cancer is lower than the amount of components in the saliva of individuals free from such tissue alterations and determining said threshold value to be the same or lower than said characteristic molecular weight.

A SIMPLE, NON-INVASIVE METHOD FOR DIAGNOSING ORAL CANCER

FIELD OF THE INVENTION

The invention relates to the field of *in vitro* diagnostics. Particularly, an *in vitro*, non-invasive method is provided for the diagnosis of head and neck or oral cancer. The method comprises quantifying a plurality of components with a molecular weight lower than a predetermined threshold value in a saliva sample obtained from a subject and comparing the so obtained quantification value to a reference value.

BACKGROUND OF THE INVENTION

Identification of potential biomarkers to detect cancer has delivered a number of candidates which may be used in early diagnosis of the disease. More recently identification of biomarker patterns, especially polypeptide patterns characteristic of cancer or a certain type of cancer has been the focus of interest. US20070087392 (WO 2005/034727) describes a method for diagnosing head and neck squamous cell carcinoma, wherein a variety of specific, individual protein biomarkers is detected. The unique protein biomarkers are differentially expressed in patients suffering from the disease. The molecular weight of the biomarkers ranges from about 2700 Da to about 15 000 Da.

Shikha Saxena et al. (A Review of Salivary Biomarker: A Tool for Early Oral Cancer Diagnosis. Adv Biomed Res. 2017; 6: 90.) have reviewed salivary biomarkers in different tumors, such as breast cancer, hepatocellular carcinoma, pancreatic cancer and oral cancer, showing that differential expression of one or more than one of the reported polypeptides may be characteristic of the given tumour.

Chaiyarit et al. Comparative evaluation of 5-15-kDa salivary proteins from patients with different oral diseases by MALDI-TOF/TOF mass spectrometry (Clin Oral Investig. 2015 Apr;19(3):729-37) have identified two mass signals (5 592,26 Da and 8301.46 Da) using MALDI-TOF/TOF mass spectrometry, which were significantly higher in oral cancer patients than in control subjects and a specific mass signal of 7.6 kDa that was increased in oral cancer patients. The authors suggest that the peptide identity of the mass signals should be determined to validate them as biomarkers suitable for detecting oral diseases. Saliva has been shown to contain multiple kinds of molecular and microbial analytes and biomarkers (Yoshizawa JM et al Salivary biomarkers: toward future clinical and diagnostic utilities. Clin Microbiol Rev. 2013 Oct;26(4):781-91.) Furthermore, whole saliva sample is easy to collect and is a promising tool in non-invasive diagnostic methods.

A number of individual proteins have been already identified as potential biomarkers for oral and head and neck cancers, which may be used alone or in combination with other potential biomarkers. The need of detecting and determining the individual biomarkers makes diagnostic methods based on individual proteins or combination of specific proteins time-consuming and costly. There is thus still a need for a diagnostic method that is simple to carry out and enables the assessment of a huge number of samples within a short time.

BRIEF DESCRIPTION OF THE INVENTION

An *in vitro*, non-invasive method is provided for diagnosing head and neck, in particular oral cancer. In the method herein provided it is not necessary to identify the chemical identity (e.g. amino acid sequence) of the

components of a saliva sample to be measured, rather the total amount of all detected components below a predetermined threshold can be used for diagnosis, without the need to differentiate between the components.

In vitro method for the detection of tissue alterations associated with a head and neck cancer in a subject, the method comprising

A) quantifying, in a saliva sample obtained from the subject,

components having a lower molecular weight than a predetermined threshold, thereby obtaining a test quantification value, and

B) identifying the subject as having a tissue alteration associated with a head and neck cancer if

the test quantification value determined in step A is higher than a reference quantification value

wherein said predetermined threshold value is obtained by determining a characteristic molecular weight above which the amount of components in the saliva of individuals having tissue alterations associated with a head and neck cancer is lower than the amount of components in the saliva of individulas free from such tissue alterations and determining said threshold value to be the same or lower than said characteristic molecular weight, and

wherein said reference quantification value is obtained by

executing step A on saliva samples obtained from an appropriate number of individuals free from said tissue alterations and determining a reference quantification value characteristic for said individuals free from said tissue alterations.

Alternatively, the predetermined threshold value is obtained by determining a characteristic molecular weight, below which the amount of the plurality of components in the saliva of subjects having tissue alterations associated with cancer is higher than in the saliva of subjects free of such tissue alterations, using samples from patients diagnosed with cancer and samples from healthy individuals (i.e. from subjects not suffering from cancer).

The reference quantification value characteristic for individuals free from tissue alterations associated with cancer may be an upper boundary value defined based on the distribution of the quantification values typical of healthy individuals. Preferably, the reference quantification value is the maximum quantification value measured in healthy individuals (i.e. in the samples used for obtaining the reference quantification value). In a preferred embodiment the reference quantification value characteristic for individuals free from tissue alterations associated with head and neck cancer is a value which is lower than the maximum quantification value, measured in healthy individuals. The reference quantification value characteristic for individuals free from tissue alterations associated with a head and neck cancer may be defined based on the distribution of the quantification values typical of healthy individuals and the distribution of the quantification values typical of head and neck cancer patients.

In preferred embodiments said threshold is determined by obtaining and analyzing mass spectra of the components present in the saliva samples of individuals having tissue alterations associated with a head and neck cancer and individuals free from such tissue alterations (see Figs. 1 and 2)

In preferred embodiments of the methods herein provided, the total amount or the total number of the mass signals (i.e. peaks) of the components having a lower molecular weight than a predetermined threshold are de-

termined.

In preferred embodiments of the methods herein provided, said predetermined threshold is from about 8000 Da to about 12000 Da, preferably about 12 000 Da, more preferably about 7000 Da to about 9 000 Da, or about 10 000 Da or about 8000 Da or about 9000 Da. In other preferred embodiments of the methods herein provided, said predetermined threshold is from about 4000 Da to about 12 000 Da, preferably from about 5 000 Da to about 10 000 Da or from about 5 000 Da to about 7 000 Da.

In preferred embodiments of the methods herein provided, the components are components which may be quantified by using UV/VIS spectrometry at a wavelength from 180 to 800 nm, preferably from 200 nm to 300 nm, more preferably from 260 nm to 300 nm, more preferably from 260 nm to 280 nm, highly preferably at about 280 nm. Preferably, the components are all the components in the saliva sample which can be detected, preferably quantified by using UV/VIS spectrometry at a wavelength from 230 to 300 nm preferably from 260 nm to 300 nm, more preferably from 260 nm to 280 nm.

In preferred embodiments of the methods herein provided, the components having a lower molecular weight than a predetermined threshold are separated using a membrane filter having an appropriate molecular weight cut-off or by a membrane filter having an appropriate pore size.

Preferably, the components having a lower molecular weight than a predetermined threshold value are quantified using absorption spectrometry, preferably UV/VIS spectrometry or infrared spectrometry, more preferably UV spectrometry.

Preferably, UV spectrometry is used at 250 nm to 300 nm, more preferably 250 nm to 280 nm, highly preferably 260 nm to 280 nm.

In particular embodiments the quantification is carried out at more than one wavelength or more than one range of a wavelength, and the quantification value is derived from the results of the measurements at the more than one wavelength or the more than one ranges of wavelength.

Provided is an *in vitro* method for the detection of tissue alterations associated with head and neck cancer in a subject, the method comprising

I)   providing a mass spectrum of a saliva sample obtained from the subject,

II)   comparing the region of the provided mass spectrum showing peak(s) of detected component(s) above a predetermined threshold value to the corresponding region of a reference mass spectrum being characteristic of individuals free from such tissue alterations, and

III)   identifying the subject as having tissue alterations associated with cancer if said region comprises less peaks than the corresponding region in the reference spectrum,

wherein said predetermined threshold value is obtained by determining a characteristic molecular weight above which the mass spectrum of the saliva of individuals having tissue alterations associated with head and neck cancer comprises less peaks than the corresponding region of the mass spectrum of saliva of individuals free of tissue alterations associated with cancer and determining said threshold value to be the same or lower than said characteristic molecular weight.

In highly preferred embodiments of the methods herein provided, the components are peptides and/or proteins. In certain embodiments of the methods herein provided, components of the saliva sample having a molecu-

lar weight higher than about 20 000 Da, preferably higher than above 18 000 Dalton are removed before carrying out step A) or I) or are not quantified or are not assessed.

In preferred embodiments of the methods herein provided, the cancer is a head and neck cancer, preferably selected from an oral cancer, parotid tumour, squamous cell carcinoma (SCC), laryngeal SCC, pharyngeal SCC, gingival SCC, supraglottic SCC, hypopharyngeal SCC, vocal chord SCC, oesophageal SCC.

In preferred embodiments a subject having a tissue alteration associated with a head and neck cancer is a subject having head and neck cancer. In these embodiments the methods herein provided are for diagnosing head and neck cancer.

In preferred embodiments the subject is a mammal, highly preferably human.

In preferred embodiments when an amount of components or a quantification value is higher or lower in a sample of a patient having a tissue alteration associated with cancer or of a patient with cancer than the amount of the plurality of components or quantification value, respectively in a reference sample, the amount is significantly higher or lower, respectively.

In a highly preferred embodiment quantifying the components having a lower molecular weight than the predetermined threshold is carried out by measuring the total absorbance of the lower molecular weight fraction of the saliva sample after separation of the components below the predetermined threshold and above the predetermined threshold.

In a highly preferred embodiment quantifying the components having a lower molecular weight than the predetermined threshold is carried out by measuring the total amount of the lower molecular weight fraction of the saliva sample after separation of the components below the predetermined threshold and above the predetermined threshold.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Representative MALDI TOF MS mass spectra of human whole saliva samples of the healthy control group

Figure 2: Representative MALDI TOF MS mass spectra of human whole saliva samples of the cancerous group

Figure 3: UV absorbance at 280 nm of saliva samples from cancer patients and healthy subjects

DETAILED DESCRIPTION OF THE INVENTION

An *in vitro* method for the detection of tissue alterations associated with head and neck or oral cancer in a subject is provided. Without wishing to be bound by theory, it is supposed that the method is suitable to detect tissue alterations associated with head and neck or oral cancer by detecting degradation products due to increased enzymatic activity involved in head and neck or oral cancer. Malignant tumours are characterised by cancer cells invading the surrounding tissues. Invasion requires the destruction of the extracellular matrix proteins by various matrix proteolytic enzymes. ECM proteins are degraded by cell-associated or extracellular enzymes secreted by both tumour cells and stromal cells.

Matrix metalloproteases (MMPs; e.g. gelatinase A, 72 kDa type-IV collagenase) degrade all of the major

components of the extracellular matrix (such as collagen IV, laminin, fibronectin, elastin, heparan-sulfate proteoglycans). Beside MMPs, serine proteases (e.g. urokinase-type plasminogen activator), heparanases (e.g. heparan sulfate proteoglycan (HSPG)), sulfatases and cystein peptidases are also implicated in the degradation of the ECM.

Proliferating altered cells (e.g. cancer cells) produce high levels of specific proteases, thereby modifying both the serum proteome and the metabolic products thereof, i.e. the serum peptidome. Components in saliva may also be derived from degradation of the extracellular matrix, altered or increased metabolism characteristic of cancer cells. Many of the peptides in the saliva are believed to be fragments of larger proteins that have been at least partially degraded by various enzymes such as metalloproteases.

The terms "quantifying", "quantify" and the like refer to measuring a characteristic of a component that may be correlated with the amount of a component. Measuring such a characteristic may be carried out by measuring an indicator of the characteristic of the component that may be correlated with the amount of the component. For example a component may be quantified by measuring the concentration of the component in a sample, wherein the concentration is measured by assessing the light absorbance of the component at a given wavelength (i.e. the absorbance is used as an indicator of the concentration).

In the method provided herein the amount of certain saliva components is determined. The term "amount of components" (in a sample) may relate to the concentration of the components in a sample or the total mass of the components (in the sample).

The amount of the components comprised in a sample may be determined by various methods. The term "determining the amount of (the) components" as used herein refers to determining and quantifying at least one characteristic of the components, which correlates with the (total) amount of the components and thus may be used as an indicator of the amount present in the sample. Such characteristics may be UV, IR, near-IR, near-UV or visible light absorbance of the components on a certain wavelength.

In certain embodiments the absolute amount of the components is determined.

In certain embodiments the amount of the components relative to the amount of other components is determined.

The components whose amount is to be determined have a molecular weight that is below or above of a predetermined threshold or cut-off value. It is within the skills of the skilled person to separate components with a molecular weight below (or above) the predetermined threshold from a sample, e.g. by using appropriate molecular sieves or membranes suitable for filtration. In certain embodiments both the amount of the components with a molecular weight below the predetermined threshold or cut-off value and the amount of the components with a molecular weight above the predetermined threshold or cut-off value are determined. In certain embodiments the quantifying of components is carried out by relating the quantity of the components to the quantity of other components. For the ease of identification, when quantifying is done by such relating, the test quantification value is termed "test relative quantification value" and the reference quantification value is termed "relative reference quantification" value herein. The (test or reference) relative quantification value (amount of the components detected) may be calculated e.g. as the total amount of all the components having a molecular weight under the predetermined threshold (detected at a certain wavelength) relative to the total amount of all the components in

the sample (detected at that wavelength). In an embodiment the relative quantification value (amount of the components detected) may be calculated as the total amount of all the components having a molecular weight under the predetermined threshold (detected at a certain wavelength) relative to the total amount of the components having a molecular weight under the predetermined threshold (detected at that wavelength). In certain embodiments the relative amount of the components detected may be calculated as the total amount of all the components having a molecular weight under the predetermined threshold (detected at a certain wavelength) to the amount of a component which is not affected by tissue alterations associated with head and neck cancer. In cases where a relative quantification value is calculated, the relative reference quantification value is obtained in the same way as the absolute reference quantification value when the absolute quantification value is measured. In preferred cases an absolute quantification value (absolute amount of the components detected) and one or more relative quantification value are used.

In certain embodiments a molecular sieve having an appropriate pore size is used to separate the components.

It is well within the knowledge and skills of the skilled person to select a filter with the appropriate molecular cut-off value or pore size. For example, when the threshold is from about 8 000 Da to about 12 000 Da or from about 8 000 Da to about 10 000 Da, filters with a pore size from about 0.45 µM to about 0.1 µM may be used.

"Test sample" or "test saliva sample" as used herein is a saliva sample gathered from a subject who is tested for having head and neck or oral cancer.

The amount of the components determined in a test sampleis compared to a reference amount, i.e. the characteristic of the component that correlates with the amount of the components and serves as an indicator of the amount of the components in the sample, is compared to the corresponding indicator of a reference sample. The reference sample is preferably from healthy subjects, i.e. individuals who are not affected by the tissue alteration associated with oral cancer. In case the reference value is from healthy subjects, the amount of the components with the lower molecular weight than the predetermined threshold value is indicative of oral cancer when said amount in the test sample is higher than in the reference sample. More than one reference values or ranges may be determined. In that case the reference values or reference ranges are correlated with healthy or cancerous conditions. The reference value can be the average or mean or the maximum value obtained from a group of healthy subjects and/or a group of subjects suffering from the cancer. The reference sample and the test sample are derived from saliva.

In certain embodiments the test sample is so prepared that components with a molecular weight above a predetermined value ("predetermined upper test molecular weight") as referred herein) are removed before carrying out the method herein provided. For example the test sample may be so prepared that components thereof having a molecular weight higher than about 20 kDa, preferably about 18 kDa, preferably about 17 kDa or components having a molecular weight higher than about 15 kDa are removed before carrying out the method. In other embodiments when the method of detection and determination is suitable, the components having a molecular weight higher than the "predetermined upper test molecular weight"are not removed from the sample before carrying out the method of the invention and only components having a molecular weight lower than the "predetermined upper test molecular weight" are examined. In preferred embodiments components with a molecular weight of below 250 Da, preferably below 500 Da are not assessed. Accordingly, only mass signals above 250

Da, preferably above 500 Da may be assessed when a mass spectrum is provided. Preferably only mass signals below 22 kDa, preferably below 20 kDa or below 18 kDa may be assessed when a mass spectrum is provided.

The term "about" as used herein and when it refers to a molecular weight value refers to a molecular weight that is in the range of ±20%, preferably in the range of ±15% of the specific value given. Thus, the term "a component with a molecular weight about 10 000 kDa" may refer to components having a molecular weight from 8 000 kDa to 12 000 kDa or from 8 500 kDa to 11 500 kDa.

The terms "tumour" and "cancer" are used interchangeably herein and refer to diseases characterized by abnormal cell growth and proliferation. The method provided is suitable to detect head and neck cancers. Head and neck cancers include oral cancers. The term "oral cancer" includes diseases characterized by any cancerous tissue growth located in the oral cavity, e.g. squamous cell carcinoma (SCC), metastatic carcinomas. The tumour is prefereably selected from parotid tumors, laryngeal SCC, pharyngeal SCC, gingival SCC, supraglottic SCC, hypopharyngeal SCC, vocal chord SCC, cervical SCC, oesophageal SCC.

The term "a plurality of" refers to more than one component. In some embodiments the total amount of the components assessed is determined, e.g. the total amount of all components below the predetermined threshold which may be quantified at the wavelength used in the quantification steps.

Threshold determination

A threshold value is determined before carrying out the method provided herein. A predetermined threshold may be obtained by determining a characteristic molecular weight above which the amount of components in the saliva of subjects having tissue alterations associated with oral cancer is lower than in the saliva of subjects free of such tissue alterations and determining said predetermined threshold value to be the same or lower than said characteristic molecular weight.

For example, mass spectra of saliva samples from tumour patients and healthy subjects – that is, subjects not suffering from cancer – are provided, e.g. by using the method described in Example 1. However, any method for providing a mass spectrum of the components in a saliva sample may be used. Based on the spectra from these two groups (oral cancer patients and healthy individuals), a characteristic molecular weight may be chosen. Components with a molecular weight above this characteristic molecular weight are missing or are present in a considerably lower amount in cancer patients than in healthy individuals. Thus, the predetermined threshold value may be the same or a lower value than the characteristic molecular weight value.

The presence of a component may be indicated by the presence of one or more peaks (i.e. a mass signal) in the mass spectrum. It is also possible to determine the amount of a component that is characterised by a certain peak on the mass spectrum by methods well known in the art, see e.g. Hiraoka: Fundamentals of Mass Spectrometry, Springer, 2013. If the amount of one or more component is determined, it is possible to compare the amount of the component(s) present in the region of the provided mass spectrum above a predetermined threshold value to the amount of component(s) present in the corresponding region of the reference spectrum. The subject is identified as having tissue alterations associated with cancer or having cancer if the amount of component(s) detected in the spectrum of the subject is less than the amount of the component(s) detected in the reference spectrum.

Reference values

One or more reference values are calculated before carrying out the method provided herein. To calculate a reference value, an appropriate number of samples are assessed, wherein the samples are obtained from an appropriate number of individuals i) free from the tissue alterations associated with oral cancer (i.e. healthy individuals), and/or ii) from an appropriate number of oral cancer patients (or patients with a tissue alteration associated with oral cancer). The skilled person is well aware of the meaning of the term "an appropriate number" when it is used in connection with a reference sample or reference value. The term "an appropriate number" refers to a plurality of samples, whose number is suitable to derive a statistically reliable and representative value from said plurality of samples. The chemical or physico-chemical properties of the components having a molecular weight below the first predetermined threshold are irrelevant as long as their molecular weight is below (or above, as the case may be) a predetermined threshold. The components may therefore be peptides, proteins, glucoproteins, lipid, triglycerids, carbohydrates or nucleic acids or any combinations thereof. The method provided herein allows fast and easy diagnosis because the components absorbing light at a certain wavelength need not to be quantified individually or separated according to their chemical properties. The total amount of all components detectable at the given wavelength is to be determined. Alternatively, the components are peptides and/or proteins, which may be detected, preferably quantified by absorbance spectrometry at 250-280 nm. Components other than peptides and/or proteins may be quantified together with the peptides and/or proteins, without distinguishing between the quantity of the components other than peptides and/or proteins and the quantity of peptides and/or proteins, i.e. the total amount of the lower molecular weight fraction may be used for identifying a subject having tissue alterations associated with head and neck cancer. However, when it may be necessary to distinguish peptides and/or proteins from other components. In these cases only the quantity of peptides and/or proteins is used for identifying a subject having tissue alterations associated with head and neck cancer.

In a preferred embodiment the components are quantified by measuring their UV and/or visible light absorbance in a spectrophotometer (UV or UV/VIS spectrometry). Another preferred embodiment uses Raman spectrometry, in particular Raman-IR spectrometry. Yet another preferred embodiment uses Fourier-transform infrared spectroscopy (FTIR). In a preferred embodiment, the wavelength used to quantify the components is 260-300 nm, highly preferably 280 nm. In other preferred embodiments, a variety of wavelengths are selected to quantify the components and the quantification value is derived from the absorbances measured at the different wavelengths. In preferred embodiments at least one of the ranges selected is 200-330 nm, preferably 220-300 nm, more preferably 260-300 nm, highly preferably a wavelength or range of wavelength at which peptides and/or proteins may be detected, e.g. about 280 nm.

The term "significantly" as used herein refers to a difference which is statistically significant. The statistical test to determine whether a difference between two values or set of values is statistically significant will depend on the characteristic measured. In other embodiments the term "significantly" refers to a difference that is being considered substantial by the skilled person. For example, when a test sample contains significantly less mass signals (peaks) or amounts of components with a molecular weight above a predetermined threshold than the mass signals of components with a molecular weight above the predetermined threshold in a reference sample, the number of mass signals or the amounts are significantly less, when the number or amount in the test sample is at least 40%, preferably at least 50%, more preferably at least 75%, most preferably at least 85% less

than the number or amount in the reference sample.

Other aspects of the invention are described herebelow.

An *in vitro* method for the detection of tissue alterations associated with a head and neck cancer in a subject,

5   the method comprising

A)   quantifying, in a saliva sample obtained from the subject,

      components having a higher molecular weight than a predetermined threshold, thereby obtaining a test
      quantification value

B)   identifying the subject as having a tissue alteration associated with a head and neck cancer if

10   the test quantification value determined in step A is lower than a reference quantification value

      wherein said predetermined threshold value is obtained by determining a characteristic molecular weight

      above which the amount of components in the saliva of individuals having tissue alterations associated with a

      head and neck cancer is lower than the amount of components in the saliva of individulas free from such tissue

      alterations and determining said threshold value to be the same or higher than said characteristic molecular

15   weight, and

      wherein said reference quantification value is obtained by

      executing step A on saliva samples obtained from an appropriate number of individuals free from said tissue

      alterations and determining a reference quantification value characteristic for said individuals free from said

      tissue alterations.

20   In preferred embodiments of the methods herein provided, said predetermined threshold is from about 4000

      Da to about 5000 Da, preferably from about 4200 Da to about 4700 Da, more preferably about 4300 Da or about

      4400 Da or about 4500 Da or about 4600 Da.

      In certain embodiments of the methods herein provided, components of the saliva sample having a molecular

      weight lower than from about 4 kDa to about 5 kDa, preferably from about 4.3 kDa to about 4.7 kDa or compo-

25   nents having a molecular weight lower than about 4.5 kDa are removed before carrying out step A) or are not

      quantified or are not assessed.

      In certain embodiments of the methods herein provided, components of the saliva sample having a molecular

      weight higher than about 20 000 Da, preferably higher than above 18 000 Dalton are removed before carrying

      out step A) or I) or are not quantified or are not assessed.

30   In certain embodiments the test sample is so prepared that components with a molecular weight below a pre-

      determined value are removed before carrying out the method herein provided. For example the test sample may

      be so prepared that components thereof having a molecular weight lower than from about 4 kDa to about 5 kDa,

      preferably from about 4.3 kDa to about 4.7 kDa or components having a molecular weight lower than about 4.5

      kDa are removed before carrying out the method. In such cases, before carrying out the method, the test sample

35   does not comprise components, e.g. peptides and proteins, having a molecular weight lower than from about 4

      kDa to about 5 kDa, preferably from about 4.3 kDa to about 4.7 kDa or components having a molecular weight

      lower than about 4.5 kDa, respectively. In other embodiments when the method of detection and determination is

      suitable, only components, e.g. peptides and proteins, having a molecular weight higher than a predetermined

molecular weight are examined and if desired, quantified. In preferred embodiments, components with a molecular weight of below from about 4 kDa to about 5 kDa, preferably below about 4.5 kDa are not quantified or not assessed. Accordingly, only mass signals above 4-5 kDa, preferably above 4.5 kDa may be assessed when a mass spectrum is provided.

Definitions, features, preferred characteristics and limitations described in respect of the other methods of the invention are meant to be applied to this aspect of the invention as well, when appropriate.

EXAMPLES

Example 1. Threshold determination (Figs. 1 and 2)

Clinical samples

Briefly, participants were asked to rinse their mouth three times thoroughly with tap water before collection of saliva samples; saliva specimen of 2 ml were taken with Braun 5 ml syringes from nonstimulated oral cavity in buccal fold and stored on ice in Protein Lobind tube (Eppendorf, Wien, Austria) using LoRetention tips (Eppendorf, Wien, Austria), followed by a 4°C centrifugation at 2500 rpm for 12 minutes. Supernatant was consequently removed and stored at minus 18° Celcius for investigation.

Mass spectrometry

The mass spectrometer used in this work was an AutoflexII TOF/TOF (Bruker Daltonics, Bremen, Germany) operated in the linear detector for MALDI TOF with an automated mode using the FlexControl software. The ions were accelerated under delayed extraction conditions (200 ns) in positive ion mode with an acceleration voltage of 20.00 kV. The instrument uses a 337 nm pulsed nitrogen laser, model MNL-205MC (LTB Lasertechnik Berlin GmbH, Berlin, Germany). External calibration was performed in each case using Bruker Peptide Calibration Standard (#206195 Peptide Calibration Standard, Bruker Daltonics, Bremen, Germany). 1 μL of the standard solutions (prepared by dissolving saturated α-cyano-4-hydroxycinnamic acid of 1.0 ml Acetonitrile/0.1% Trifluoracetonic acid 50/50 (V/V)) were loaded onto the target plate (MTP 384 target plate ground steel TF, Bruker Daltonics, Bremen, Germany)

The extracts of the saliva in Protein Lobind tube (Eppendorf, Wien, Austria) were loaded onto the target plate using LoRetention tips (Eppendorf, Wien, Austria) by mixing 1.0 μL of each solution with the same volume of a matrix solution, prepared by dissolving saturated Sinapic acid of 1.0 ml Acetonitrile/0.1% Trifluoracetonic acid 50/50 (V/V))

Each spectrum was processed by accumulating data from 500 consecutive laser shots for Peptide calibration standard solution and 1000 shots for saliva samples. The Bruker FlexControl 2.4 software was used for controlling the instrument and the Bruker FlexAnalysis 2.4 software for spectra evaluation. Protein masses were acquired with a range of m/z 5000 to m/z 18000.

Results

Participants were divided into a cancer (diagnosed with oral cancer, SCC) and a healthy (i.e. subjects not suffering from cancer) control group. Each group contained a total of n=50 participants.

Spectra of m/z 5000 to m/z 18000 were regarded with MALDI TOF evaluation. Spectra were divided in excerpts concerning m/z 6000-8000, m/z 9000-12000 and m/z 14000-16000 in each group.

Healthy control group showed peaks in each segment. A definite presence of components, specifically at m/z 15552 (±28) and m/z 14325 (±33) in the high mass protein segment appeared (Figure 1).

The middle segment in this group showed two peaks between m/z 9617 and m/z 11207 and a further two peaked segment in between m/z 7628 and m/z 5800.

In the cancer group, changes were visible at ranges from m/z 6000 to m/z 9104 where a complete disappearance of higher mass proteins over expression was discovered. Peaks in lower protein mass segment were considerably at two mass counts differing in all regarded patients of between m/z 6639 and m/z 7494 (Figure 2).

Significant changes could be identified between control and cancerous saliva specimen. Changes in protein levels between m/z 13000 to m/z 16000 were developing to a total disappearance in cancer patients, whereas the healthy control group showed a definite appearance of those proteins. Protein levels in the range of m/z 9000 to m/z 12000 were at a minimum in cancerous individuals. The spectrum of small sized proteins in levels between m/z 6000 to m/z 8000 were significantly higher in saliva of tumorous patients. Our results show a decrease of molecular weight proteins above 9 kDa to 18 kDa.

Enzymatic cellular internal digestion or overexpression of enzymatic activity might be responsible reasons for the disappearing of (overexpressed) proteins with masses above 9 kDa and could state the higher count of lower mass proteins below 9 kDa. Higher enzymatic activity has been stated to be represented in cancer cells.

The developed method is already suitable clinically to separate cancer patients from healthy condition, as well as it is cheap and noninvasive and suitable for a high-throughput method with estimated 5000 possible samples per day.

Example 2. UV and/or UV-VIS spectrophotometry

Clinical samples

Briefly, participants (patients with an oral cancer diagnosis and healthy subjects (i.e. subjects not suffering from cancer)) were asked to rinse their mouth three times thoroughly with tap water be-fore collection of saliva samples; saliva specimen of 2 ml were taken with Braun 5 ml syringes from nonstimulated oral cavity in buccal fold and stored on ice in Protein Lobind tube (Eppendorf, Wien, Austria) using LoRetention tips (Eppendorf, Wien, Austria).

Spectrophotometry

One milliliter of unstimulated saliva samples were filtered by a syringe filter membrane device with 10 kDa nominal molecular weight cut off and/or 0.1 micrometer pore size. The supernatants were collected directly into a plastic 10 mm / 1mm micro cuvette (UVette, light transmission of 200 nm – 1600 nm, Eppendorf, Wien, Austria). The spectrophotometer used in this work was an Eppendorf BioSpectrometer D30. The absorbance spectra were acquired 200-830 nm. The contents of the saliva samples were determined at 280 nm with factory setting of protein mode. Each sample was measured three times and three technical replicates were produced from each collected saliva.

Results are shown in table 1.

Table 1 UV absorbance of the assessed saliva samples (absorbance units)

Tumour	Healthy
2.51	0.85

2.24	1.00
3.00	0.68
3.00	0.47
3.00	0.91
3.00	0.83
3.00	1.58
2.92	1.75
2.30	1.60
3.00	0.35
2.92	0.18
3.00	0.43
3.00	0.51
3.00	0.35
3.00	0.60
2.48	0.74
2.87	0.35
3.00	
3.00	
3.00	
3.00	
3.00	
2.78	
2.22	
3.00	
3.00	
3.00	
3.00	
3.00	
2.10	
3.00	
3.00	
3.00	
2.82	
2.68	
3.00	
3.00	
3.00	

3.00
3.00
3.00
2.22
3.00
3.00
2.73
2.52
3.00
3.00

CLAIMS

1. *In vitro* method for the detection of tissue alterations associated with a head and neck cancer in a subject, the method comprising

A) quantifying, in a saliva sample obtained from the subject,

components having a lower molecular weight than a predetermined threshold, thereby obtaining a test quantification value, and

B) identifying the subject as having a tissue alteration associated with a head and neck cancer if

the test quantification value determined in step A is higher than a reference quantification value,

wherein said predetermined threshold value is obtained by determining a characteristic molecular weight above which the amount of components in the saliva of individuals having tissue alterations associated with a head and neck cancer is lower than the amount of components in the saliva of individulas free from such tissue alterations and determining said threshold value to be the same or lower than said characteristic molecular weight, and

wherein said test reference quantification value is obtained by

executing step A on saliva samples obtained from an appropriate number of individuals free from said tissue alterations and determining a reference quantification value characteristic for said individuals free from said tissue alterations.

2. The method according to claim 1, wherein the components are all the components in the saliva sample which can be detected, preferably quantified, by using UV/VIS spectrometry at a wavelength from 230 to 300 nm, preferably from 250 nm to 300 nm, more preferably from 250 nm to 280 nm and highly preferably from 260 nm to 280.

3. The method according to claim 1 or 2, wherein said predetermined threshold value is determined by obtaining and analyzing mass spectra of the components present in the saliva samples of individuals having tissue alterations associated with a head and neck cancer and individuals free from such tissue alterations.

4. The method according to any one of the preceding claims, wherein the components having a lower molecular weight than a predetermined threshold value are separated using a filter having an appropriate molecular weight cut-off or having an appropriate pore size.

5. The method according to any one of the preceding claims, wherein the components having a lower molecular weight than a predetermined threshold value are quantified using absorption spectrometry, preferably UV/VIS spectrometry or infrared spectrometry, more preferably UV spectrometry.

6. The method according to claim 5, wherein quantification is carried out using UV spectrometry at a wavelength of 230 to 300 nm, preferably from 250 nm to 300 nm, more preferably from 250 nm to 280 nm and highly

preferably from 260 nm to 280.

7. *In vitro* method for the detection of tissue alterations associated with a head and neck cancer in a subject, the method comprising

I)   providing a mass spectrum of a saliva sample obtained from the subject,

II)   comparing the region of the provided mass spectrum showing peak(s) of detected component(s) above a predetermined threshold value to the corresponding region of a reference mass spectrum being characteristic of individuals free from such tissue alterations, and

III)   identifying the subject as having tissue alterations associated with a head and neck cancer if said region comprises less peaks than the corresponding region in the reference spectrum,

wherein said predetermined threshold value is obtained by

determining a characteristic molecular weight above which the mass spectra of the saliva of individuals having tissue alterations associated with head and neck cancer comprise less peaks than the corresponding region of the mass spectra of saliva of individuals free from such tissue alterations and

determining said threshold value to be the same or lower than said characteristic molecular weight.

8. The method according to any one of the preceding claims, wherein said predetermined threshold value is in the range of 7 000 Da to 12 000 Da, preferably in the range of 7 000 Da to 10 000 Da and highly preferably in the range of 8 000 Da to 10 000 Da.

9. The method according to any one of the preceding claims, wherein components of the saliva sample having a molecular weight higher than 20 000 Da, preferably higher than 18 000 Dalton are removed before carrying out step A) or I) or are not assessed.

10. The method according to any one of the preceding claims, wherein the head and neck cancer cancer is , selected from an oral cancer, parotid tumour, squamous cell carcinoma (SCC), laryngeal SCC, pharyngeal SCC, gingival SCC, supraglottic SCC, hypopharyngeal SCC, vocal chord SCC, oesophageal SCC.
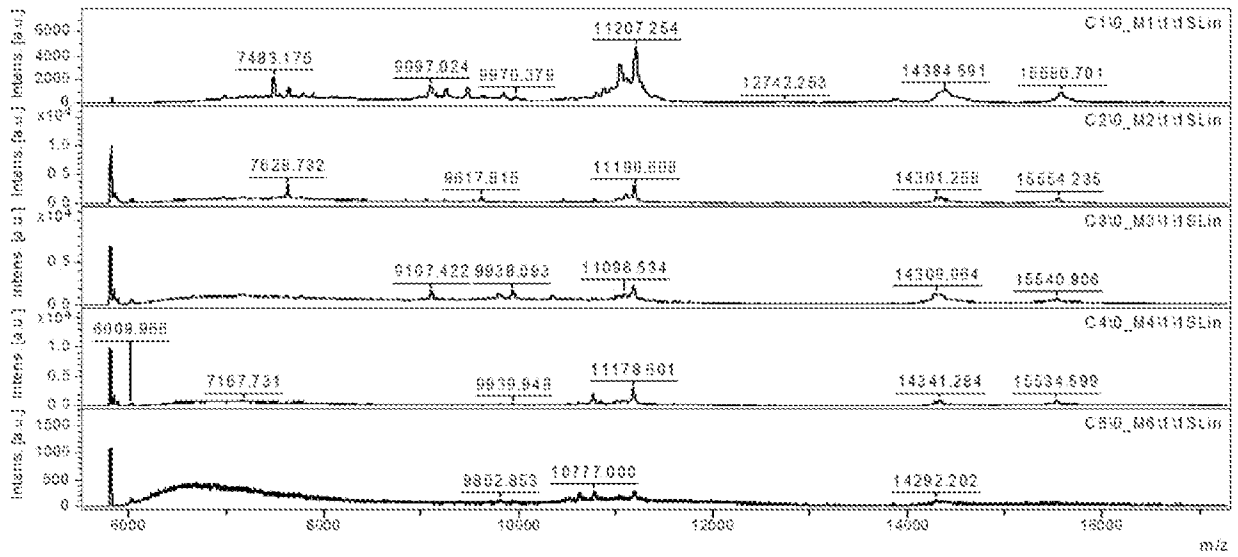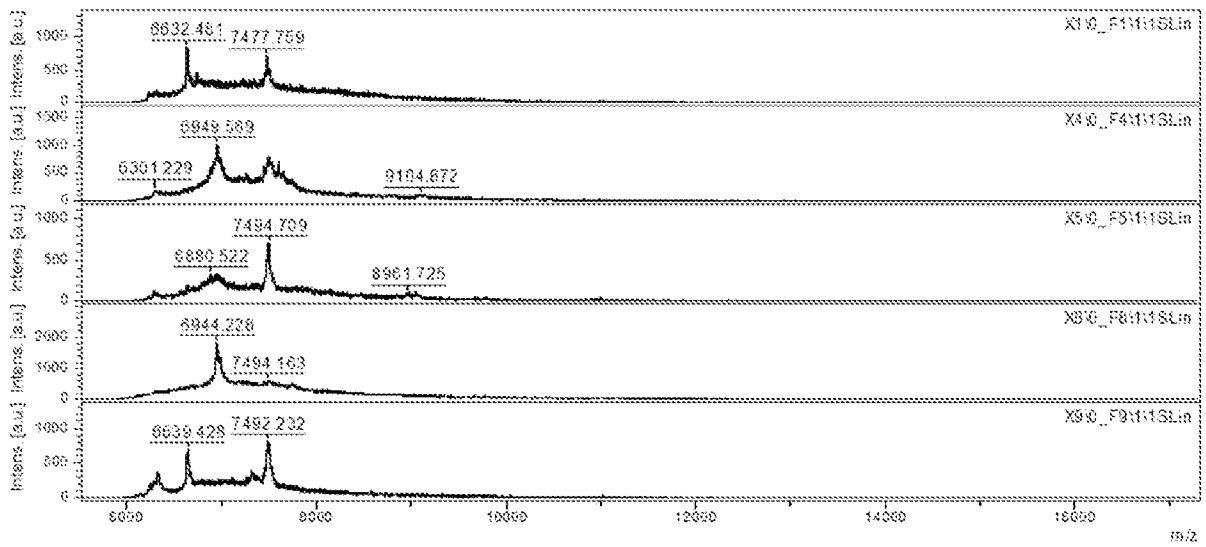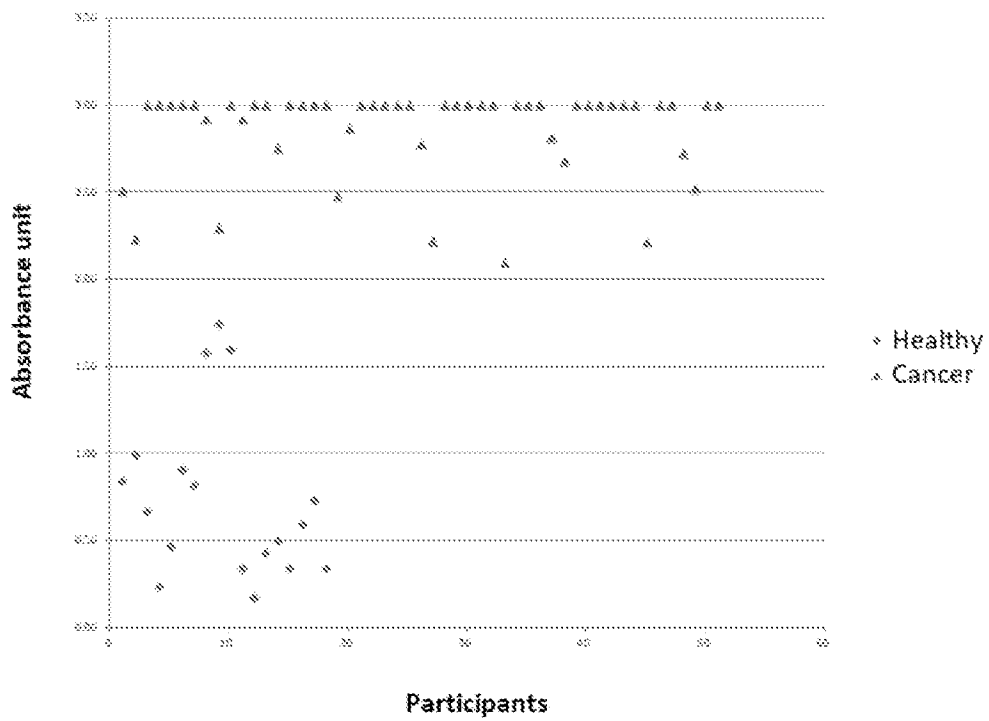
Figure 1

Figure 2

Figure 3

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/574   C12Q1/6886
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 C12Q

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, WPI Data

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| [X] | Further documents are listed in the continuation of Box C. | | [X] | 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	Date of mailing of the international search report
6 March 2020	17/03/2020

| Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016 | Authorized officer Boiangiu, Clara |

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