

A METHOD OF DETECTING HFMD

The present invention relates to a method of detecting Hand-Foot-and-Mouth Disease (HFMD) in samples obtained from test subjects. In particular, the method relates to the
5 detection of protein biomarkers in the saliva of these test subjects.

HFMD, a seemingly unassuming acute infectious disease which is typically mild and self-limiting, is a widespread epidemic viral disease which afflicts millions of infants and children yearly in the Western Pacific region caused by the human enterovirus species A (HEV-A) from
10 the genus *Enterovirus*. Till recent years, Coxsackievirus A16 (CA16) and Enterovirus 71 (EV71) were the principal etiological factors of HFMD (Wang and Liu, 2014). However, the surge in the number of cases caused by other HEV-A serotypes such as Coxsackievirus A6 (CA6) was also reported. HFMD is customarily a self-limiting disease characterized by fever and
15 papulovesicular, sometimes maculopapular, rash on the palms, soles, elbows, and trunk as well as mouth ulcers. However, EV71-associated HFMD can quickly develop into severe neurological complications such as aseptic meningitis and acute flaccid meningitis in a modest proportion of cases. These neurological complications may in turn swiftly progress to cardiopulmonary failure and mortality. Even though neurologic complications have been
largely associated with EV71, and also been reported to cause neurological complications.
20 The various complications and manifestations that could arise from enterovirus infections strongly necessitate a rapid and accurate identification of enterovirus so that efficient isolation of infected patients could be carried out to prevent further spreading.

HFMD is rapidly transmitted either via faecal-oral or droplet route and is currently diagnosed
25 by physicians via clinical symptoms and manifestations. Additional laboratory testing is mostly deemed unnecessary for mild cases. Nevertheless, the aforementioned can lead to misdiagnosis and could aggravate spreading of HFMD in atypical and mild cases. In addition, there is currently no cure for HFMD. Treatment options are confined to alleviating of physical symptoms. Therefore, rapid and accurate diagnosis spanning a range of etiological agents
30 causing HFMD becomes critical when there is a risk of neurological complication leading to fatality.

The golden criterion of laboratory HFMD diagnosis is the identification of virus isolates from clinical samples such as throat or epidermal vesicle swab. The enterovirus could be isolated in human muscle rhabdomyosarcoma (RD) cells and African green monkey kidney (Vero) cells and subsequently could be subjected to reverse-transcription polymerase chain reaction (PCR) of viral RNA, indirect immunofluorescence and viral microneutralization assays. However, the abovementioned approaches are rather lengthy and time-consuming. Although rapid diagnostic methods utilizing modern molecular routines such as quantitative real-time PCR (qRT-PCR) were recently developed to address those issues, the sensitivity of such assay needs significant improvement due to diverse genetic differences between serotypes of enteroviruses.

Besides non-existent cross-protective prophylactic multi-valent vaccines and effective broad-spectrum anti-viral drugs, the lack of standardised and accurate diagnostic approaches also contribute to the unnecessary and potentially exorbitant socio-economical, financial and/or psychological burdens of this highly transmissible infectious disease. Due to limited laboratory infrastructure and support on-sites, HFMD diagnosis in day-care centres, schools, communities, institutions, and outpatient settings is often crudely dependent on its classical clinical features. However, visual judgements inevitably result in misdiagnoses as a result of diagnostic complications arising from non-distinctive or atypical clinical manifestations. Consequently, imprecise diagnostic discrimination result in inappropriate medical regimen or uncontrollable disease transmission.

Therefore, the development of HFMD point-of-care testing (POCT) is imperative for pertinent therapeutic judgments as well as timely clinical isolation and treatment regime for the actual cause. Tapping on virus dynamics is a crucial bottleneck in a complete diagnostic coverage of the non-exhaustive spectrum of HFMD-causing viruses. As such, moving towards virus-specific systemic host signatures may be a promising alternative in the development of POC diagnostics for the multi-causative disease.

Therefore, there is a need for an improved method for detecting the viruses that cause HFMD.

The listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

- 5 Any document referred to herein is hereby incorporated by reference in its entirety.

The fight against hand, foot and mouth disease (HFMD) remains an arduous challenge without existing point-of-care diagnostic platforms for accurate diagnosis and prompt case quarantine. Hence, the purpose of this salivary biomarker discovery study is to set
10 the fundamentals for the realisation of POC diagnostics for HFMD.

Advantageously, the present invention employed a reductive demethylation (R-diMe) chemical labelling method coupled with high resolution mass spectrometry (MS)-based quantitative proteomics technology to screen the entire salivary proteome for commonly
15 dysregulated proteins during HFMD. The shortlisted candidates were further assessed for its potential as HFMD salivary diagnostic biomarkers. As such, the present invention are directed toward these protein biomarkers for the detection of HFMD infection in a sample obtained from an individual.

20 In an aspect of the present invention, there is provided a method for determining hand-foot-and-mouth disease (HFMD), the method comprising detecting in a saliva sample the presence and/or amount of at least one protein biomarker selected from the group defined in Table 1 (as shown in Figure 4), wherein the presence of the protein biomarker in the saliva sample is indicative of HFMD.

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In various embodiments, the method of the present invention includes measuring the amount of the protein biomarker present in the sample. For example, the amount may be measured by measuring the intensity of the band or dots detected by a colorimetric assay carried out by a person skilled in the art. In addition, the results obtained may be compared against
30 positive and negative control samples.

The present invention serves to detect the presence and/or amount of said protein biomarkers in saliva because obtaining a saliva sample involves non-invasive techniques which will facilitate the ease of collection from patients such as infants, toddlers and young children that form the majority of those affected and afflicted by HFMD. Given the quick spread of the disease among in schools and at day-care centres, it is important that these infants, toddlers and young children be screened easily and quickly on a daily basis so that any infection can be detected quickly before the disease spreads to others in the schools or day-care centres. A quick diagnosis can allow the infected child to be removed from the school population, so that the spread of the disease is minimised or arrested. This means that it would be advantageous to have a non-invasive method of detecting the disease. The present invention offers such an advantage by allowing the detection of the disease using saliva samples obtained from the children. The saliva samples do not require any processing prior to carrying out the present detection method. In addition, the present method need not be performed by medical professionals but instead can be performed and carried out by parents and school teachers easily and readily, i.e. point of care testing. In contrast, the collection of blood for HFMD diagnosis in young children is challenging and needs to be performed under clinical conditions.

By "biomarkers", it is meant to include any molecular indicators of a specific biological property, a biochemical feature or facet that can be used to measure the progress of disease or the effects of treatment. Proteins and nucleic acids are exemplary biomarkers. In particular, it has been accepted that genomic messengers detected extracellularly can serve as biomarkers for diseases. In particular, nucleic acids have been identified in most bodily fluids including blood, urine and cerebrospinal fluid, and have been successfully adopted for using as diagnostic biomarkers for diseases. However, saliva is not a passive "ultrafiltrate" of serum, but contains a distinctive composition of enzymes, hormones, antibodies, and other molecules. Specific and informative biomarkers in saliva are desirable to serve for diagnosing disease and monitoring human health. For example biomarkers have been identified in saliva for monitoring caries, periodontitis, oral cancer and salivary gland diseases.

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Preferably, the protein biomarkers is selected from the group consisting of PRX-IV, legumain, SIL1 and CREG1, and detecting the presence and/or amount of either one or any combination

of said biomarkers in the saliva sample is indicative of HFMD. The present method allows for the detection of HFMD by detecting not only the individual protein biomarkers but also in combination of any one of them.

5 In various embodiments, the step of detecting the presence and/or amount of the at least one protein biomarker is performed using a binding agent capable of binding to the at least one protein biomarker to form a bound complex. Suitable binding agents include any agent selected or screened from a library based on their ability to bind to the protein biomarkers of the present invention set out in Table 1. For example, the binding agent may be any peptide
10 binding molecule, which works similarly like the antibodies described in this application that binds to the biomarker protein. Any such binding or hybridisation method may be used that is known to a person skilled in molecular biology techniques. Such agents may be any given nucleic acid, protein or amino acid motif suitable to bind to any one of the present protein biomarkers. These may include any antibody or a fragment thereof.

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In various embodiments, the antibody may be any anti-human antibody (for example, anti-IgG, -IgM, -IgA etc.).

In various embodiments, examples of such antibodies include any anti-PRX-IV, anti-legumain,
20 anti-SIL1 and anti-CREG1 antibodies.

The antibodies may be monoclonal or polyclonal. Suitable monoclonal antibodies may be prepared by known techniques, for example those disclosed in "Monoclonal Antibodies: A manual of techniques", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and applications", J G R Hurrell (CRC Press, 1982), both of which are incorporated
25 herein by reference. A fragment may contain one or more of the variable heavy (VH) or variable light (VL) domains. For example, the term antibody fragment includes Fab-like molecules (Better et al (1988) Science 240, 1041); Fv molecules (Skerra et al (1988) Science 240, 1038); single-chain Fv (ScFv) molecules where the VH and VL partner domains are linked
30 via a flexible oligopeptide (Bird et al (1988) Science 242, 423; Huston et al (1988) Proc. Natl. Acad. Sci. USA 85, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward et al (1989) Nature 341, 544). It also includes any "antibody variant" which means any

synthetic antibodies, recombinant antibodies or antibody hybrids, such as but not limited to, a single-chain antibody molecule produced by phage-display of immunoglobulin light and/or heavy chain variable and/or constant regions, or other immunointeractive molecule capable of binding to an antigen in an immunoassay format that is known to those skilled in the art.

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A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) Nature 349, 293-299.

10 In various embodiments, it is possible for the binding agent to be an aptamer.

Molecular libraries such as antibody libraries (Clackson et al, 1991 , Nature 352, 624-628; Marks et al, 1991 , J Mol Biol 222(3): 581-97), peptide libraries (Smith, 1985, Science 228(4705): 1315-7), expressed cDNA libraries (Santi et al (2000) J Mol Biol 296(2): 497- 508),
15 libraries on other scaffolds than the antibody framework such as affibodies (Gunneriusson et al, 1999, Appl Environ Microbiol 65(9): 4134-40) or libraries based on aptamers (Kenan et al, 1999, Methods Mol Biol 118, 217-31) may be used as a source from which binding agents that are specific for a given motif are selected for use in the methods of the present invention, particularly those that would adhere and bind to those protein biomarkers defined in Table
20 1.

The molecular libraries may be expressed in vivo in prokaryotic (Clackson et al, 1991 , op. c/f.; Marks et al, 1991 , op. cit.) or eukaryotic cells (Kieckhefer et al, 1999, Proc Natl Acad Sci USA, 96(10):5651-6) or may be expressed in vitro without involvement of cells (Hanes & Pluckthun, 1997, Proc Natl Acad Sci USA 94(10):4937-42; He & Taussig, 1997, Nucleic Acids Res 25(24):5132-4; Nemoto et al, 1997, FEBS Lett, 414(2):405-8). In cases when protein based
25 libraries are used often the genes encoding the libraries of potential binding molecules are packaged in viruses and the potential binding molecule is displayed at the surface of the virus (Clackson et al, 1991 , op. cit; Marks et al, 1991 , op. cit; Smith, 1985, op. cit).

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When potential binding molecules are selected from libraries one or a few selector peptides having defined motifs are usually employed. Amino acid residues that provide structure,

decreasing flexibility in the peptide or charged, polar or hydrophobic side chains allowing interaction with the binding molecule may be used in the design of motifs for selector peptides.

5 As will be described in detailed, in various embodiments, the method of the present invention is carried out by any suitable arrays. In the case of a lateral flow assay, a primary antibody is immobilised to a substrate (e.g. membrane) or matrix to capture the biomarker protein. A capture antibody is then allowed to bind to the biomarker protein, the capture antibody is coupled to an enzyme that changes colour upon capturing of the protein biomarker.

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As such, in various embodiments, the at least one biomarker in the test sample is labelled with a detectable moiety. The detectable moiety may be selected from the group consisting of: a fluorescent moiety, a luminescent moiety, a chemiluminescent moiety, a radioactive moiety, and an enzymatic moiety.

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By a "detectable moiety", it is meant to include any moiety which may be detected and the relative amount and/or location of the moiety determined. A detectable moiety may be a fluorescent and/or luminescent and/or chemiluminescent moiety which, when exposed to specific conditions, may be detected. For example, a fluorescent moiety may need to be
20 exposed to radiation (i.e. light) at a specific wavelength and intensity to cause excitation of the fluorescent moiety, thereby enabling it to emit detectable fluorescence at a specific wavelength that may be detected.

In various embodiments, the detectable moiety is a gold colloid. Alternatively, or in addition,
25 the antibodies used in the present invention may be conjugated with gold colloid or gold nanoparticles.

Alternatively, the detectable moiety may be an enzyme which is capable of converting a (preferably undetectable) substrate into a detectable product that can be visualised and/or detected. Examples of suitable enzymes used may be those known for use in assays such as
30 the ELISA.

Alternatively, the detectable moiety may be a radioactive atom which is useful in imaging. Suitable radioactive atoms include ^{99m}Tc and ^{123}I for scintigraphic studies. Other readily detectable moieties include, for example, spin labels for magnetic resonance imaging (MRI) such as ^{123}I again, ^{131}I , ^{111}In , ^{19}F , ^{13}C , ^{15}N , ^{17}O , gadolinium, manganese or iron. Clearly, the agent to
5 be detected (such as, for example, the one or more proteins in the test sample and/or control sample described herein and/or an antibody molecule for use in detecting a selected protein) must have sufficient of the appropriate atomic isotopes in order for the detectable moiety to be readily detectable.

10 The radio- or other labels may be incorporated into the agents of the invention (i.e. the proteins present in the samples of the methods of the invention and/or the binding agents of the invention) in known ways. For example, if the binding moiety is a polypeptide it may be biosynthesised or may be synthesised by chemical amino acid synthesis using suitable amino acid precursors involving, for example, fluorine-19 in place of hydrogen. Labels such
15 as ^{99m}Tc , ^{123}I , ^{186}Rh , ^{188}Rh and ^{111}In can, for example, be attached via cysteine residues in the binding moiety. Yttrium-90 can be attached via a lysine residue. The IODOGEN method (Fraker et al (1978) Biochem. Biophys. Res. Comm. 80, 49-57) can be used to incorporate ^{123}I . Reference ("Monoclonal Antibodies in Immunoscintigraphy", J-F Chatal, CRC Press, 1989) describes other methods in detail. Methods for conjugating other detectable moieties (such
20 as enzymatic, fluorescent, luminescent, chemiluminescent or radioactive moieties) to proteins are well known in the art.

In various embodiments, the step of detecting the presence and/or amount of the at least one protein biomarker is performed using a lateral flow assay. Such assays are simple
25 cellulose-based devices intended to detect the presence (or absence) of a target analyte in liquid sample (matrix), saliva in the present case, without the need for specialized and costly equipment. Typically, these tests are used for medical diagnostics either for home testing, point of care testing, or laboratory use. The technology is based on a series of capillary beds, such as pieces of porous paper, micro-structured polymer, or sintered polymer. Each of
30 these elements has the capacity to transport the saliva fluid spontaneously.

A first element (the saliva sample pad) acts as a sponge and holds an excess of sample fluid. Once soaked, the fluid migrates to a second element ("anti-protein biomarker" conjugate pad) in which the anti-protein biomarkers are bound, a dried format of bio-active particles in a salt-sugar matrix that contains everything to guarantee an optimised chemical reaction
5 between the protein biomarkers (if present in the saliva sample) and its chemical partner (e.g., anti-protein biomarkers) that has been immobilised on the particle's surface. While the sample fluid dissolves the salt-sugar matrix, it also dissolves the particles, and in one combined transport action, the sample and conjugate mix while flowing through the porous structure. In this way, the analyte binds to the particles while migrating further through the
10 third capillary bed. This material has one or more areas (often called stripes) where a third molecule may be immobilized by the manufacturer. By the time the sample-conjugate mix reaches these strips, analyte has been bound on the particle and the third "capture" molecule binds the complex. After a while, when more and more fluid has passed the stripes, particles accumulate and the stripe-area changes colour. Typically, there are at least two stripes: one
15 (the control) that captures any particle and thereby shows that reaction conditions and technology worked fine and one that contains a specific capture molecule and only captures those particles onto which an analyte molecule has been immobilized. After passing these reaction zones, the fluid enters the final porous material, the wick, that simply acts as a waste container.

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In various embodiment, the method of the present invention may be carried out in a reverse lateral flow assay. In such an assay, the method comprises (a) providing a solid support having a first end and a second end; (b) immobilising a binding agent (for example, an antibody described in the present invention) on the solid support, the binding agent may comprise
25 more than one and, if so, they are immobilised separately and spaced apart from each other along the solid support; (c) immobilising a molecule on the second end of the solid support, the molecule (capture molecule such as a further antibody) capable of binding to the antibody in the complex, the molecule further comprising at least one detectable labelled moiety; (c) loading the sample (e.g. salivary sample containing possible protein biomarkers set out in
30 Table 1) on the first end of the solid support, the sample contacting the binding agent(s) as it travels along the solid support in a first direction along the solid support and towards the second end; and (d) loading a buffer on the second end of the solid support adjacent the

molecule such that the buffer solution solubilise the molecule as it travels along the solid support in a second direction opposite the first direction, wherein the presence of the complex formed on the solid support is detected by the labelled moiety of the molecule. The detectable labelled moiety may be a gold conjugate.

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Such a method may further provide for an absorbent pad that is placed adjacent to or abuts the first end of the solid support, the absorbent pad and the solid support are separated by a separator, wherein removing the separator allows contact between the absorbent pad and the solid support to enhance the flow of the buffer along the solid support in the second
10 direction, for example to increase the flow rate of the buffer.

Any such binding method may be used that is known to a person skilled in immunoassay techniques.

15 The lateral flow assays described above may be put into the form of a kit (e.g. POCT kit). The kit may have one or more reaction chambers such that a plurality of samples may be run alongside each other in a single reaction.

In various embodiments, the amount of the at least one protein biomarker present in the
20 range of between 0.1ug to 1mg indicates a HFMD infection.

Preferably, the detectable moiety is a stable isotope or radioactive moiety. The at least one protein biomarker is then detected using a high resolution mass spectrometry.

25 In another aspect of the present invention, there is provided a method for aiding in categorising, diagnosing or determining prognosis in a patient with HFMD, the method comprising comprising detecting in a saliva sample the presence of at least one protein biomarker selected from the group defined in Table 1, wherein the presence of the protein biomarker in the saliva sample is indicative of HFMD.

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In another aspect of the present invention, there is provided the use of one or more protein biomarkers selected from the group defined in Table 1 as a biomarker for determining HFMD in a human individual.

- 5 In yet another aspect of the present invention, there is provided a kit for diagnosing HFMD in a human individual, the kit comprising an assay for detecting in a saliva sample the presence of at least one protein biomarker selected from the group defined in Table 1.

The kit may further include water and hybridization buffer to facilitate hybridisation of the
10 binding agent with the protein biomarkers that may be present in a sample

The components of the kits may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably,
15 suitably aliquoted. Where there is more than one component in the kit (labeling reagent and label may be packaged together), the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. The kits of the present invention also will typically include a means for containing the nucleic acids, and any
20 other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained. When the components of the kit are provided in one and/or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred.

25 However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

30 The container means will generally include at least one vial, test tube, flask, bottle, syringe and/or other container means, into which the nucleic acid formulations are placed,

preferably, suitably allocated. The kits may also comprise a second container means for containing a sterile, pharmaceutically acceptable buffer and/or other diluent.

5 The kits of the present invention will also typically include a means for containing the vials in close confinement for commercial sale, such as, e.g., injection and/or blow- molded plastic containers into which the desired vials are retained.

Such kits may also include components that preserve or maintain the oligonucleotides or that protect against its degradation. Such components may be RNase-free or protect against
10 RNAses. Such kits generally will comprise, in suitable means, distinct containers for each individual reagent or solution. A kit will also include instructions for employing the kit components as well the use of any other reagent not included in the kit. Instructions may include variations that can be implemented.

15 It is contemplated that such reagents are embodiments of kits of the invention. Such kits, however, are not limited to the particular items identified above and may include any reagent used for the manipulation or characterization of the protein markers. In various embodiments, the kit and assay method of the present invention may include those disclosed in US 6,316,205.

20 Advantageously, the present invention's easy and straightforward collection method omits sample processing and does not require any technical expertise to operate, thereby facilitating the realism of point-of-care outside of hospitals and laboratories. Since only saliva is needed for diagnosis, our proposed diagnostic kit could potentially be used in households,
25 day-care centres, schools, communities, institutions, and outpatient settings whereby laboratory infrastructure and support are not readily available. The input volume of saliva required for the test is as low as 5µl, which allows for potential development.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the
30 claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

It is contemplated that any embodiment discussed herein can be implemented with respect to any method or composition of the invention, and vice versa. Furthermore, compositions and kits of the invention can be used to achieve methods of the invention.

- 5 Throughout this application, the term "about" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure
10 supports a definition that refers to only alternatives and "and/or."

As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or
15 "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed
20 description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

25 In order that the present invention may be fully understood and readily put into practical effect, there shall now be described by way of non-limitative examples only preferred embodiments of the present invention, the description being with reference to the accompanying illustrative figures.

30 In the Figures:

Figure 1 is a volcano plot of HFMD salivary biomarker candidates.

Figure 2 is a chart showing normalised dot intensities of dysregulated salivary proteins in HFMD and 219 healthy cases. The left box in each chart represents “Enterovirus negative”, while the right box in each chart represents “Enterovirus positive”. The dot intensities of each case was normalised using the included normaliser 220 in each blot for subsequent comparison across blots. Mann-Whitney non-parametric test was 221 performed to compare the dot intensities of salivary PRX-IV, legumain, SIL, and CREG1 in 222 HFMD and healthy cases. *** p value < 0.001.

Figure 3 is a ROC curve of dysregulated salivary proteins for the prediction of HFMD 226 status. Salivary PRX-IV, legumain, SIL1, and CREG1 had an AUC of 0.775, 0.755, 0.550, 227 and 0.518 respectively. This suggested relatively good discriminating tests for PRX-IV and 228 legumain, but non-discriminating tests for SIL1 and CREG1.

Figure 4 shows Table 1 shows the list of 19 up-regulated (shaded) and 51 down-regulated (non-shaded) proteins of the present invention. The relative expression of salivary proteins in HFMD cases (normalised to healthy cases) were recorded. Average fold changes and p values were calculated based on the data obtained from the three sets of biological replicates (D64, D66, and D68). Salivary proteins with average fold change ≥ 1.5 (p value ≤ 0.05) were shown in the table.

Figure 5 is a schematic workflow for laboratory-based confirmatory 16 diagnosis of HFMD cases.

Figure 6 is a schematic diagram for the screening of HFMD salivary 20 biomarker. Equal amount of pooled patient salivary proteins (n = 3) and pooled healthy 21 salivary proteins from volunteers (n = 3) were processed using dimethyl-label gel-free MS, 22 detect for differentially-expressed proteins between the two groups (i.e. HFMD vs. healthy).

Figure 7 is a schematic diagram for the validation of HFMD candidate 26 salivary biomarkers using dot blot assay.

Figure 8 shows Table 2: Assessment of candidate salivary biomarkers.

Figure 9 shows Table 3: Assessment of combinatory candidate salivary biomarkers using radial
5 basis 236 function kernel function analyses.

Example 1

1. Materials and Method

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Whole salivary proteome profiling was performed on the saliva obtained from children with HFMD and healthy children, using a reductive dimethylation chemical labelling method coupled with high resolution mass spectrometry-based quantitative proteomics technology.

15

The comprehensive protocols for the recruitment of HFMD patients from KK Women's and Children's Hospital and healthy volunteers from child-care centres were approved by and carried out under the guidelines of the Ethics Committee of the SingHealth CIRB (reference number: 2012/448/E) and NUS-IRB (reference code: B-14-273) respectively.

20

The patients were evaluated by clinicians using a clinical case definition of fever, oral ulcers, and skin lesions on the palms and soles for HFMD. The clinically-defined HFMD cases were confirmed using viral genotyping. The healthy volunteers were tested negative for enteroviruses as well (see Figure 5).

25

Pooled saliva from laboratory-confirmed HFMD cases were compared to that from laboratory-excluded healthy cases using dimethyl-label gel-free MS. Briefly, the protein samples were denatured in 8M urea, reduced in 5mM DTT, and alkylated in 10mM IAA. Subsequently, in-solution digestion was performed using Lys-C (1:100) at
30 37°C O/N under strong denaturing conditions (6M urea), followed by trypsin (1:50) at 37°C for 4h under weaker denaturing conditions (1M urea). The peptides of the test and control groups were then labelled with different isotopomers on their primary

amines through R-diMe to yield different pools of labelled peptides. The differentially labelled samples were mixed in 1:1 ratio and fractionated into 12 fractions using the 3100 OFFGEL fractionator (Agilent Technologies, USA). Individual fractions were finally concentrated and desalted using self-assembled C₁₈ stage tips before elution for MS analysis (see Figure 6). The relative signal intensities of each peptide pair ('heavy' vs. 'light') were quantitated in the same MS spectrum in an unbiased manner, to determine the relative abundances of each identified protein. The MS data was analysed by MaxQuant version 1.3.0.5 using the Uniprot Human FASTA database, where maximum false discovery rates were set to 0.01. Protein identities were supported by at least one unique peptide with a minimum length of seven amino acids.

Proteins with peptide/ratio counts of less than 2, common MS contaminants, and proteins identified only by sites were excluded from the output list. Three biological replicates were performed, and the output lists were combined in Mascot software to detect for commonly dysregulated proteins during HFMD. Proteins that were at least 1.5-fold differentially expressed with p value of less than 0.05 were selected from the combined list.

For dot blot validation, 5µl of saliva samples were dotted onto a 0.2µm nitrocellulose membrane. A normaliser was added to each membrane to account for Western blot film exposure differences across blots. A total of 36 diseased and 46 healthy saliva samples were used for validation. The samples were allowed to be adsorbed for 1h. The membranes were rinsed with PBS thrice before probing for the respective protein candidates using Western blot. The primary antibodies used were anti-legumain EPR14718 (#ab183028), anti-peroxiredoxin 4 EPR15458 (B) (#ab184167), anti-SIL1 (#ab5639), and anti-CREG1 AT1C6 (#ab201699) from Abcam (UK). The secondary antibodies used were anti-rabbit IgG-HRP (#NA934V) and anti-mouse IgG-HRP (#NA931) from GE Healthcare (USA), as well as anti-goat IgG-HRP (#sc-2020) from Santa Cruz Biotechnology, Inc. (USA). The relative dot intensities were measured using Image J program (National Institutes of Health, USA) and normalised to the normaliser included in each blot (see Figure 7). The normalised dot intensities of the respective salivary proteins were compared between HFMD patients and healthy volunteers. All statistical

analyses were performed using GraphPad Prism version 4.0 (GraphPad software, USA), using Mann-Whitney non-parametric tests (without Gaussian distribution assumption). p-values below 0.05 were considered to be statistically significant.

5 2. Results

The salivary proteome of HFMD patients was compared to that of healthy 125 volunteers using R-diMe labelling coupled with gel-free MS. The volcano plot in Figure 1 showed the commonly dysregulated proteins during HFMD across three biological
10 replicates. The dots with protein identity represent proteins which were significantly up-regulated while the dots without protein identity represent proteins which were significantly down-regulated. 19 up-regulated and 51 down-regulated proteins were found to be statistically significant (set out in Table 1 shown in Figure 4).

15 Up-regulated proteins were chosen for downstream validation. Extensive online database literature search was performed to eliminate hits that were reportedly associated with oral diseases such as periodontitis and oral cancers. Proteins which have low or no expression in saliva or salivary glands were also excluded from the validation list. The final shortlisted hits, namely peroxiredoxin-4 (PRX-IV), legumain, SIL1, and CREG1,
20 were chosen based on literature and database search exclusion as well as novelty.

Dot blot validation assay was performed using saliva samples from HFMD-diagnosed patients and healthy volunteers to assess the sensitivity and specificity of the respective biomarker(s). As normality testing performed prior to data analyses revealed that the data
25 was not normally-distributed, Mann-Whitney non-parametric tests were performed for subsequent statistical comparisons.

Mann-Whitney non-parametric tests revealed that salivary PRX-IV ($p < 0.001$) and legumain ($p < 0.001$), but not SIL1 ($p = 0.446$) and CREG1 ($p = 0.790$), were significantly
30 augmented in HFMD cases. The median dot intensities of salivary PRX-IV in HFMD and healthy cases were 9,927 (min - max: 3,102 - 21,600) and 7,268 (min - max: 2,504 - 12,550) respectively. The median dot intensities of salivary legumain in HFMD and healthy cases

were 4,965 (min- max: 178.2 - 13,240) and 1,765 (min - max: 399.6 - 8,874) respectively. The median dot intensities of salivary SIL1 in HFMD and healthy cases were 8,127 (min - max: 2,603 -28,670) and 9,286 (min - max: 1,518 - 16,890) respectively. The median dot intensities of salivary CREG1 in HFMD and healthy cases were 9,176 (min - max: 2,507 - 34,250) and 10,420 (min - max: 1,896 - 15,510) respectively (see Figure 2; and Figure 8 Table 2).

The ROC curves of the respective proteins also demonstrated the prognostic utility of salivary PRX-IV and legumain, but not SIL1 and CREG1, in distinguishing between HFMD and healthy cases. The ROC of salivary PRX-IV had an AUC of 0.775 (95% CI: 0.668-0.883, $p < 0.001$). The ROC of salivary legumain had an AUC of 0.755 (95% CI: 0.646-0.865, $p < 0.001$). The ROC of salivary SIL1 had an AUC of 0.550 (95% CI: 0.419 - 0.680, $p = 0.444$). The ROC of salivary CREG1 had an AUC of 0.518 (95% CI: 0.387 - 0.648, $p = 0.786$) (see Figure 3; and Figure 8 Table 1). Radial basis function kernel function analyses further revealed that the AUC of the best performing single candidate could be improved to 0.813 when used in combination with salivary SIL1 (as shown in Figure 9 Table 3).

3. Discussion

In the present investigation, HFMD patient salivary proteome was screened for dysregulated salivary proteins during HFMD. Herein, we identified 70 HFMD-dysregulated salivary proteins, where 19 of them were up-regulated while 51 of them were down-regulated. Four proteins, namely PRX-IV, legumain, SIL1, and CREG1, were chosen for downstream validation. PRX-IV is a positive regulator of NF- κ B through modulation of IKK-mediated phosphorylation of inhibitory $1 \kappa B\alpha$. This is an interesting finding as NF- κ B signalling, which has myriad anti-viral roles, is often a viral target to escape host immunity. Many studies have reported attenuated NF- κ B activity, both directly and indirectly by various enterovirus non-structural proteins. However, Sauter et al (2015) reported the possibility of both pro- and anti- viral roles in NF- κ B.

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Legumain is a cysteine protease involved in degradation of internalized EGFRs as well as exogenous lysosomal antigen processing pathway for MHC class II presentation.

Virus protein processing in the lysosomes could be a prerequisite for viral antigen presentation by MHC class II molecules for an immune response. Indeed, MHC class II deficiencies were reported to be associated with increased susceptibility for enterovirus infections.

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SIL1, a nucleotide-exchange factor that regulates the ATPase activity of Hsp70 Bip chaperon, is required for protein folding and assembly as well as protein shuttling in the ER. This protein could be partly involved in extensive ER remodelling, which is a prerequisite for the formation of virus-induced membranous structures for virus replication. These structures, also known as the viral RC, not only concentrate essential host factors to facilitate efficient virus replication, but also encase and shield viral components against host immunity.

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Protein CREG1 is a secreted glycoprotein involved in the negative transcriptional regulation of cell growth and differentiation.

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The validation data showed that the relative expressions of salivary PRX-IV and legumain, consistent with the screening data, were significantly augmented in HFMD cases.

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Diagnostic sensitivity and specificity of the prospective salivary proteins were also evaluated. While high sensitivity is indispensable for screening to identify diseased individuals, high specificity is also imperative for diagnosis to differentiate healthy individuals from diseased individuals. However, sensitivity and specificity can be arbitrary based on a compromised criterion determined by the observer. Hence, ROC curve analyses, a popular evaluation method for clinical diagnostic tests, were performed to assess the prognostic utility of these proteins as HFMD biomarkers instead. The ROC curve plots true positives (sensitivity) against false positives ($1 - \text{specificity}$), to derive different possible combinations of sensitivity/specificity thresholds of a prognostic test.

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Hence, the AUC is a useful and unbiased assessment of the ability of the predictor as a diagnostic tool to discern diseased state from healthy state. It measures the discriminative power of the prospective biomarker, where an AUC of 1 indicates a

perfect diagnostic test while an AUC of 0.5 indicates a non-discriminating diagnostic test [49]. The analyses confirmed the prognostic utility of salivary PRX-IV and legumain in distinguishing between HFMD and healthy cases. Further analyses also revealed the potential of using combinatory biomarker candidates for enhanced diagnostics.

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The UC - ROC curve is a performance measurement for classification problem at various thresholds settings. ROC is a probability curve and AUC represents degree or measure of separability. It tells how much model is capable of distinguishing between classes. Higher the AUC, better the model is at predicting 0s as 0s and 1s as 1s. By analogy, Higher the AUC, better the model is at distinguishing between patients with disease and no disease. Example of calculation given here: <https://towardsdatascience.com/understanding-auc-roc-curve-68b2303cc9c5>.

The present invention illustrates the potential use of saliva for HFMD POC diagnostics based on host proteome changes. As the main targeted end users are young children, this pilot study overcomes the current limitations of the existing diagnostic testing which is either clinical- or molecular-based. The non-invasive nature of the collection process is aimed at receptiveness of diagnostic testing. The easy and straightforward collection method, which omits sample processing and does not require any technical expertise to operate, is intended to facilitate the realism of POCT outside of hospitals and laboratories. Since saliva is a systemic representative of the human body, its use in POCT will enable timely detection and isolation of HFMD cases.

Whilst there has been described in the foregoing description preferred embodiments of the present invention, it will be understood by those skilled in the technology concerned that many variations or modifications in details of design or construction may be made without departing from the present invention.