Agonists of Orally Expressed TRP Channels Stimulate Salivary Secretion and Modify the Salivary Proteome

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In Brief

Quantitative proteomics revealed sources of variation and differentially secreted proteins in saliva after TRP channel agonist mouth rinsing. Menthol and nonivamide mouth rinsing increased saliva flow rates and Cystatin S was increased in nonivamide stimulated saliva. TRPM8 and TRPV1 agonists have potential in treating chronically dry mouth.

Graphical Abstract
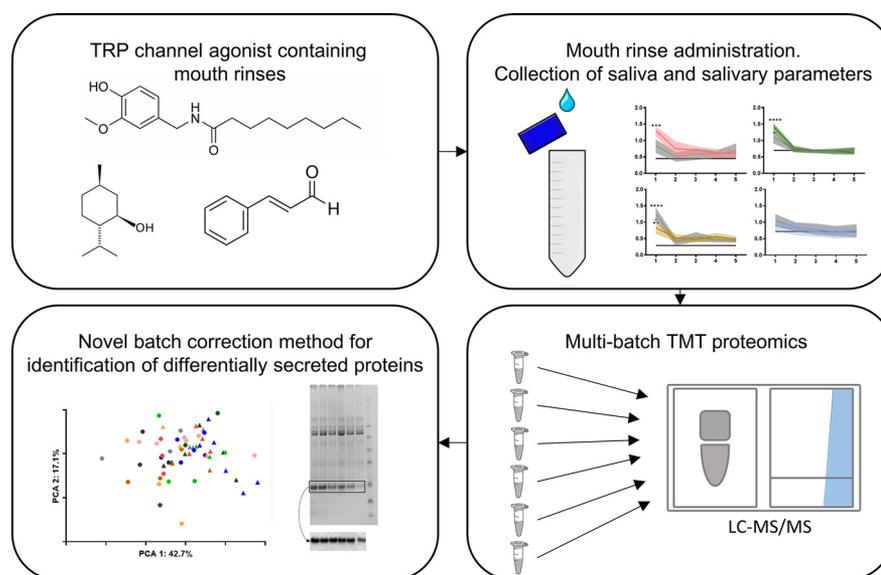
Highlights

- Salivary secretion was increased by mouth rinsing with TRP channel agonists.

- The salivary proteome varied over time and was changed by TRP channel stimulation.

- Immunoreactive Cystatin S was increased in saliva after TRPV1 stimulation.

Agonists of Orally Expressed TRP Channels Stimulate Salivary Secretion and Modify the Salivary Proteome

Jack William Houghton[1,2,*] , **Guy Carpenter**[2] , **Joachim Hans**[3], **Manuel Pesaro**[3], **Steven Lynham**[4], and **Gordon Proctor**[2]

Natural compounds that can stimulate salivary secretion are of interest in developing treatments for xerostomia, the perception of a dry mouth, that affects between 10 and 30% of the adult and elderly population. Chemesthetic transient receptor potential (TRP) channels are expressed in the surface of the oral mucosa. The TRPV1 agonists capsaicin and piperine have been shown to increase salivary flow when introduced into the oral cavity but the sialogogic properties of other TRP channel agonists have not been investigated. In this study we have determined the influence of different TRP channel agonists on the flow and protein composition of saliva. Mouth rinsing with the TRPV1 agonist nonivamide or menthol, a TRPM8 agonist, increased whole mouth saliva (WMS) flow and total protein secretion compared with unstimulated saliva, the vehicle control mouth rinse or cinnamaldehyde, a TRPA1 agonist. Nonivamide also increased the flow of labial minor gland saliva but parotid saliva flow rate was not increased. The influence of TRP channel agonists on the composition and function of the salivary proteome was investigated using a multi-batch quantitative MS method novel to salivary proteomics. Inter-personal and inter-mouth rinse variation was observed in the secreted proteomes and, using a novel bioinformatics method, inter-day variation was identified with some of the mouth rinses. Significant changes in specific salivary proteins were identified after all mouth rinses. In the case of nonivamide, these changes were attributed to functional shifts in the WMS secreted, primarily the over representation of salivary and nonsalivary cystatins which was confirmed by immunoassay. This study provides new evidence of the impact of TRP channel agonists on the salivary proteome and the stimulation of salivary secretion by a TRPM8 channel agonist, which suggests that TRP channel agonists are potential candidates for developing treatments for sufferers of xerostomia.

TRP (Transient Receptor Potential) channels are a superfamily of nonselective cation channels that respond to a variety of somatosensory and endogenous stimuli. TRPV1, 3, 4, TRPA1 and TRPM8 are expressed in the oral cavity that have thermo- and chemoreceptive functions. They are expressed on mucosal and epithelial free afferent nerve endings of myelinated Aδ and nonmyelinated C fibers (1), oral epithelial cells (2–4), taste buds (5, 6), and keratinocytes (7).

Cinnamaldehyde is a TRPA1 agonist, which is produced synthetically and found in cinnamon, a spice that comes from the bark of cinnamon trees (8). Cinnamaldehyde makes up 90% of the essential oil extracted from cinnamon bark. On contact, cinnamaldehyde provokes a feeling of warmth (8) and has potential anti-inflammatory (9–11) and anti-cancer (12–18) properties . Menthol is a TRPM8 agonist that provokes a cooling sensation. It is found in mint leaves and produced synthetically (19). Nonivamide is a capsaicinoid that elicits a burning sensation (20). It is structurally very similar to the more widely studied TRPV1 agonist capsaicin and is naturally found in chili peppers or produced synthetically.

The salivary response to basic tastants is well studied but the salivary response to TRP channel agonists requires further investigation. Increased salivary flow rate and specific protein secretion have been demonstrated in response to other tastants (21–24) and there are studies demonstrating increases in salivary flow rates and specific protein changes in response to the TRPV1 agonists (25–29) but there has been limited study of agonists to other TRP channels, despite expression of these channels in the oral cavity, nor has the mechanism of TRP channel agonist stimulated salivary secretion been elucidated.

Studying compounds that can stimulate salivary flow is of interest to the development of treatments for xerostomia, the perception of a dry mouth, that affects between 10 and 30% of the adult and elderly populations (30, 31). Acidic tastants

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that strongly stimulate salivary secretion erode enamel tissues, so alternative molecules are sought (32). Although xerostomia is often associated with hyposalivation, where the WMS flow rate is reduced by ~50% (33), this is not always the case (34). Xerostomia in the absence of hyposalivation may be because of changes in the interaction of saliva with oral surfaces because of the altered integrity of salivary proteins (35) or changes in saliva rheology (36). There is evidence that TRP agonists modify the rheological properties of saliva but the mechanism by which these changes occur remains to be elucidated. Taken together, identifying compounds that not only induce salivary secretion but also modify the rheological properties of saliva is of interest to developing treatments for xerostomia.

Specific protein changes in saliva in response to differing stimuli are possible because of the many sources of proteins which are likely to respond differently to different nerve mediated stimuli. For example, the submandibular and sublingual glands secrete in response to olfaction (37) whereas the parotid glands do not (38). Conversely, the parotid glands are preferentially stimulated by chewing which results in a higher amylase output (39). In these scenarios, proteins associated with specific glands, *e.g.* higher amylase secretion by the parotid glands or mucin secretion by the submandibular and sublingual glands, will have a relatively increased abundance when compared with unstimulated levels.

The regulation of specific proteins separate from preferential gland stimulation has also been reported. Annexin A1 and calgranulin A are up-regulated in WMS through an inflammatory-like response after mouth rinsing with bitter, umami and sour tastants (40). Bader *et al.* demonstrated the up-regulation of lysozyme in saliva stimulated by citric acid rinse (41). The TRPV1 agonist 6-gingerol up-regulated salivary sulfhydryl oxidase 1 resulting in reduced 2-furfurylthiol levels in exhaled breath and thus reduction in the perceived sulfur-like after-smell (42). However, the mechanism of these specific protein upregulations has not been elucidated.

The present study is formed of two parts. A bottom-up quantitative proteomics study of the salivas secreted by two participants in response to menthol, cinnamaldehyde, nonivamide and propylene glycol (PG) that were compared with unstimulated saliva using MS. In addition, data on WMS flow rates and protein output were also collected. In order to improve the identification of lower abundance salivary proteins, a method novel to salivary proteomics was used. Secondly, studies were conducted to confirm the specific protein changes of the proteomes of salivas identified in the proteomics study and to consider the mechanism by which the compounds exert their effects on the salivary proteomes.

EXPERIMENTAL PROCEDURES

Experimental Design and Statistical Rationale—For the proteomics study, the proteome of 60 WMS samples, obtained from two male volunteers of ages 24 and 27, were analyzed by TMT quantita-

TABLE I
Demographic information of participants in the WMS study

Study	Mean Age	Sem	n	Male	Female
Nonivamide	25.3	2.1	7	4	3
Menthol	27.2	1.5	6	3	3
Cinnamaldehyde	27.6	4.1	6	3	3
PG	27.2	2.5	6	3	3

TABLE II
The concentrations of mouth rinses used in each saliva collection of the proteomics study. Each collection consisted of an unstimulated saliva sample, followed by a 30 s mouth rinse and then 2 × 1-min post-mouth rinse saliva samples. Each collection was carried out in triplicate for two participants, totaling 48 collections. The compound, concentration and PG content in each of the mouth rinses used for this study are shown in the table

Compound	Concentration (Ppm)	PG Dilution
PG	1.8×10^4	n/a
PG	3.0×10^4	n/a
Menthol	300	6.0×10^3 ppm
Menthol	500	1.0×10^4 ppm
Cinnamaldehyde	180	1.8×10^4 ppm
Cinnamaldehyde	300	3.0×10^4 ppm
Nonivamide	0.6	6.0×10^2 ppm
Nonivamide	1.0	1.0×10^3 ppm

tive MS. Forty eight experimental samples consisting of WMS produced after mouth rinsing were split randomly across six TMT10plex batches with each batch containing two controls consisting of pooled unstimulated saliva from each participant. The 48 WMS samples were collected from two participants after being exposed to eight conditions each with three experimental repeats. In a further study of the effects of agonists on WMS secretion, 25 participants were recruited (the demographic information of each participant group is shown in Table I) six of these subjects also participated with further participants in the following studies. For the parotid saliva study, eight volunteers were recruited (38.7 ± 5.3 years, male $n = 4$, female $n = 4$). For the lower labial gland saliva study, ten volunteers were recruited (29.4 ± 4.7 years, male $n = 5$, female $n = 5$). For all studies, volunteers were healthy individuals recruited by internal advertisement with the following exclusion criteria: on prescription medication, age > 65years or < 18years, suffering from oral discomfort. The controls and statistical tests used for each analysis are described below.

Proteomics Study of TRP Agonist Stimulation on Two Subjects— Forty eight saliva collections were made in total, each collection including an unstimulated saliva sample, followed by a mouth rinse and then two post-mouth rinse saliva samples (Table II). Eight different mouth rinse solutions were tested in triplicate: nonivamide, cinnamaldehyde, menthol and PG (Symrise AG) (Table II). The solutions were prepared in pre-weighed universal tubes and the total weight recorded. The compounds were diluted in water (Buxton, UK) on the day of collection and were stored at room temperature. Participants were asked not to consume food, water or smoke in the 1 h before collection. The following guidance was given to each participant before each collection: tilt your head slightly forward to allow saliva to pool underneath the tongue; do not move your mouth unless it is to spit out collected saliva; spit out whenever it is comfortable; do

not swallow. For each collection, the following protocol was adhered to: One minute of unstimulated WMS was collected in a pre-weighed universal tube; 10 ml of mouth rinse was then taken into the mouth for 30 s and spit back into a pre-weighed universal tube; two, 1 min collections of post-mouth rinse WMS in pre-weighed universal tubes. Immediately after collection, participants were asked, "How would you rate the intensity of the mouth rinse" and were asked to give a rating from 0 – 10 on a visual analogue scale alongside an oral description of their perception of the mouth rinse. One collection was carried out per day at 2 pm and the order of mouth rinses were randomized for each participant. All samples were weighed in the universal tube straight after collection. Saliva was then processed for storage before MS analysis: samples were transferred to ice cooled 1.5 ml microtube for centrifugation (13,500 rpm, 5 min, 4 °C). Supernatants were removed, frozen at −20 °C and finally moved to −80 °C storage; the pellets were discarded.

WMS Saliva Collection

Effects of TRP Agonists on WMS Flow Rates—Cinnamaldehyde, menthol, and nonivamide were obtained from Symrise AG, Germany and prepared in PG. 300 ppm cinnamaldehyde, 500 ppm menthol, 1 ppm nonivamide, and 3×10^4 ppm PG were prepared by diluting in water (Buxton) in pre-weighed universal tubes and the total weights were recorded. The concentration of PG in the nonivamide, menthol and cinnamaldehyde mouth rinses was 3×10^3, 1×10^4, and 3×10^4 ppm respectively. The solutions were kept at room temperature (20 °C). Participants were asked not to consume food, water or smoke in the 1 h before collection. Before collection each participant was asked to tilt their head slightly forward to allow saliva to pool underneath the tongue, to not move their mouth unless it was to spit out collected saliva, to spit out whenever it is comfortable and to not swallow. Five minutes of unstimulated WMS was collected in a pre-weighed universal tube as a control. Ten ml of a control mouth rinse containing either the equivalent concentration of PG as in the TRP agonist containing mouth rinse or water was then taken into the mouth for 30 s and spat back into a pre-weighed universal tube, this was followed by five 1 min collections of WMS into pre-weighed universal tubes. This was repeated with the experimental mouth rinse. All samples were weighed in the universal tube immediately after collection. Samples were kept on ice after collection. The neat saliva samples were aliquoted into 2 ml microtubes and then centrifuged (13 500 rpm, 4 °C, 5 min). The supernatant was removed, aliquoted and stored at −20 °C.

Parotid Saliva Collection—Five 10 ml solutions were prepared: water (Buxton); propylene glycol (3.0×10^4 ppm), menthol (100 ppm), cinnamaldehyde (60 ppm), nonivamide (1 ppm). These solutions were prepared in pre-weighed universal tubes and the total weights recorded. The solutions were kept at room temperature (20 °C). Lashley cups were fitted over the exit of the Stenson's ducts, secured and correct fitting was tested by the administration of a few drops of 2% citric acid onto the tongue to stimulate parotid secretion. Time was allowed so that the collection tubes of the Lashley tubes were filled with parotid saliva. Before collection each participant was asked to not swish any solution around in their mouth in order to prevent Lashley cups being dislodged. The volunteer was given 10 ml water to practice holding the solution in the mouth and spitting it out. Unstimulated parotid saliva was collected in a pre-weighed universal tube for 5 min. Ten ml of water (Buxton) was then taken into the mouth and held for 5 min. During this time parotid saliva was collected in a pre-weighed universal tube. This was repeated with the control and TRP agonist solutions in the following order: propylene glycol, menthol, cinnamaldehyde, and nonivamide. A 2 min break was taken between each solution. Saliva samples were kept on ice after collection. The neat saliva samples were aliquoted into 2 ml microtubes and then centrifuged (13,500 rpm, 4 °C, 5 min). The supernatant was removed, aliquoted and stored at −20 °C.

Lower Labial Gland Saliva Collection—A cotton roll was placed over each Stenson duct's papilla and under the tongue to absorb major gland saliva. The inferior labial surface was dried, and unstimulated lower labial saliva was allowed to bead on the surface of the inferior labium for 2 min. A 2 cm x 1 cm piece of pre-weighed Whatman's (General Electric) filter paper was then placed on the lower labial surface with one of the 1 cm edges halfway down the midpoint of the inferior labium to collect the beads of saliva. The saliva-soaked filter paper was placed in a pre-weighed 1.5 ml microtube, weighed and the flow rate calculated by subtraction of the pre-weighed paper and pre-weighed microtube weights and divided by the time of collection in minutes. To allow for slight variations in the size of the filter paper, flow rates were scaled according to the mass of the dried filter paper. This process was repeated but with a 30 s mouth rinse of either 3.0×10^4 ppm PG, 300 ppm cinnamaldehyde, 500 ppm menthol or 1 ppm nonivamide being administered before the drying of the inferior labium. The following guidance was given to each participant before collection: ensure the mouth rinse baths the surface of your lower lip; do not swallow the mouth rinse. A 3 min break, or until the perception of the previous mouth rinse had diminished, was taken between each solution. Saliva infused filter paper samples were kept on ice after collection.

Saliva infused filter paper was placed into 0.5 ml microtubes that had 4 needle-sized holes pierced into their underside. Each 0.5 ml microtube was then placed into a 1.5 ml microtube and centrifuged (13,000 rpm, 4 °C, 5 min). The saliva collected in the 1.5 ml microtube was immediately processed for SDS-PAGE (see below) with the following modification: the entire volume of the collected saliva (~1 µL) was treated with 10 µL lithium dodecyl sulfate (LDS) sample buffer and 1 µL DTT (DTT) before heating and electrophoresis.

Quantitative Tandem Mass Spectrometry—The first minute and second minute post-mouth rinse samples from each collection were pooled. The 24 unstimulated samples from each of the two participants (48 in total) were pooled into two unstimulated pools, one for each participant. Five µL of each pooled sample was added to 95 µL PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) for protein quantification using a Bradford assay (Thermo Scientific). Absorbance of each sample was read by spectrophotometer at 595 nm and compared with a standard curve of BSA of known protein concentration. Fifty µg of protein was extracted from each sample and frozen at −80 °C. Frozen samples were freeze dried and reconstituted in 70 µL 100 mM triethylammonium bicarbonate (TEAB) and 0.1% sodium dodecyl sulfate (SDS). 10 µL 8 mM tris (2-carboxyethyl) phosphine (TCEP) in 100 mM TEAB, 0.1% SDS was added to each sample and incubated at 55 °C for one hour. 10 µL 375 mM iodoacetamide (IAA) in 100 mM TEAB, 0.1% SDS was added to each sample and incubated at room temperature for 30 min. 4 µL of 0.25 µg/µL trypsin (Roche, sequencing grade) was added to each sample and left overnight at 37 °C.

Forty one µL of TMT reagent was added to each of the 48 post mouth rinse samples and the 12 unstimulated pool samples (see Table III for details) and incubated at room temperature for 1 h. Eight µL of 5% hydroxylamine was added to each sample and left at room temperature for 15 min. Samples from each 10plex batch were pooled into six 10plex sample pools and stored at −80 °C before freeze drying until completion.

IEF fractionation was carried out using the Agilent 3100 OFFGEL system (Agilent Technologies Inc, Germany) and was carried out according to the manufacturers protocol. 1.8 ml OFFGEL buffer stock added to each sample for reconstitution. Six OFFGEL strips with a linear pH gradient ranging from 3 to 10, one for each 10plex

TABLE III
Quantitative analysis of the salivary proteome: TMT 10plex batch information. P, pool

TMT Label	Compound	Concentration (Ppm)	TMT 10plex 1	TMT 10plex 2	TMT 10plex 3	TMT 10plex 4	TMT 10plex 5	TMT 10plex 6
					Sample ID (Participant #.Repeat #)			
126	Unstimulated	na	1.P	1.P	1.P	1.P	1.P	1.P
127_N	Unstimulated	na	2.P	2.P	2.P	2.P	2.P	2.P
127_C	Cinnamaldehyde	180	2.2	1.2	1.3	2.1	1.1	2.3
128_N	Nonivamide	0.6	1.2	2.1	1.1	2.2	2.3	1.3
128_C	Cinnamaldehyde	300	1.1	1.3	2.1	2.2	1.2	2.3
129_N	PG	3.0×10^4	1.1	2.2	2.3	1.2	1.3	2.1
129_C	Menthol	500	2.3	1.2	1.3	2.2	2.1	1.1
130_N	PG	1.8×10^4	2.2	2.1	1.3	1.1	2.3	2.1
130_C	Menthol	300	2.2	1.2	2.1	1.3	1.1	2.3
131	Nonivamide	1	1.2	2.1	1.3	2.2	1.1	2.3

sample pool, were hydrated in 50 μL OFFEGL rehydration solution for 15 min. 12-fraction frames were fitted to each of the strips and 150 μL of reconstituted sample loaded into each fraction well. IEF was carried out under the following conditions: 20 kVh (100 h, V: 500-5400 V, max. I: 50 μA. On completion, each fraction was removed and frozen at −80 °C. Fractions were thawed on ice and pooled into six fraction pools (Fraction 1 with 7, 2 with 8, 3 with 9, 4 with 10, 5 with 11 and 6 with 12). Ten μL of elution buffer (50% acetonitrile (ACN), 0.1% formic acid) was added to each sample. Zip-Tips were hydrated twice in 10 μL hydration solution (50% ACN, trifluoroacetic acid (TFA)) and then washed in 1 μL of wash solution (0.1% TFA). S10 μL samples was washed through the Zip-Tip 10 times before eluting with elution solution (0.1% TFA). The elute was frozen at −80 °C before freeze drying until completion. Fractions were reconstituted in 10 μL 50 mM ammonium bicarbonate. The peptides from each fraction were resolved using reverse-phase chromatography on a 75 μM C18 EASY column using a 3-step gradient of 5–40% ACN and a 95% ACN wash in 0.1% formic acid at a rate of 300 μL/min over 220 min (EASY-NanoLC, ThermoScientific). Nano-ESI was performed directly from the column and ions were analyzed by using an LTQ Orbitrap Velos Pro (ThermoScientific). Ions were analyzed using a Top-10 data-dependent switching mode with the 10 most intense ions selected for HCD for peptide identification and reporter ion fragmentation in the Orbitrap. Automatic gain control targets were 30,000 for the iontrap and 1,000,000 for the orbitrap

Quantitative MS Data Analysis—Tandem mass spectra were extracted from the Xcalibur data system (version 2.2, ThermoScientific) and searched through Mascot (v. 2.6.0) using Proteome Discoverer software (version 1.4.0.288, ThermoScientific) to determine specific peptides and proteins. The parameters included: 20 ppm peptide precursor mass tolerance; 0.5 Da for the fragment mass tolerance; 2 missed cleavages, trypsin enzyme; TMT-6plex (N terminus and K), carbamidomethyl (C) and oxidation (M) dynamic modifications; database: UniProt_HUMAN (release-2018_02, 20 366 entries). False discovery rate was set at 0.05 and 0.01 for relaxed and strict parameters respectively, with validation based on q-Value. The data were analyzed using KNIME and embedded R scripts (KNIME analytics platform, Germany). Peptides were excluded from analysis if they were unassigned or had missing TMT channel intensity data; the primary accession number was taken for each peptide and proteins were grouped by this accession number with the geomean of individual peptide intensities given as the protein intensity value; TMT intensities were normalized using a sum scaling method and to the geomean of the two standard values for each peptide. Batches were then concatenated, batch corrected using ComBat (43) and PCA, clustering (XMeans and k-Means), gene ontology (GO) and specific

protein analyses (fold changes and TTests) were carried out. Venn diagrams were produced using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/). As the ComBat algorithm is only applicable to proteins present in all batches, a novel method of comparing samples across batches was developed. PCA plots of each nonComBat corrected batch were carried out separately and Euclidean distances between each post-mouth rinse sample and the relevant unstimulated pool calculated. These Euclidean distances were then expressed relative to the distance between the two unstimulated pools which are present in each batch and, in theory, will vary to the same degree in each batch (supplemental Fig. A).

Total Protein Concentration Assay—The total protein concentration of collected saliva samples were determined by bicinchoninic acid assay (Thermo Scientific). Frozen saliva samples were defrosted on ice and then diluted 1:10 in ddH2O in duplicate alongside a serial dilution of BSA standard (2 mg/ml - 0.03125 mg/ml). Samples and standards were incubated with bicinchonic acid for 30 min before measuring absorbance as 540 nm using an iMark microplate absorbance reader (Bio-Rad).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—Sodium dodecyl sulfate PAGE (SDS-PAGE) was carried out on saliva samples. Saliva samples were prepared for electrophoresis by dilution 4 × concentration LDS sample buffer (Invitrogen) with the addition of 0.5M DTT (Sigma) to the sample-buffer solution and then boiled for 3 min. Pre-cast 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen) were assembled in a XCell vertical electrophoresis unit (Invitrogen) with MES running buffer (Invitrogen). Samples were loaded with equal protein concentration and electrophoresed for 32 min at 125 mA and 200 V (constant). Molecular masses were determined by comparison with SeeBlue Plus2 standard proteins (Thermo Scientific).

Glycoprotein Staining—Polyacrylamide gels were placed in 0.2% Coomassie Brilliant Blue R250 in 25% methanol and 10% acetic acid at room temperature for 90 min, followed by overnight de-staining in 10% acetic acid. Periodic acid Schiff's (PAS) staining: 60 min fixing in 25% methanol and 10% acetic acid, incubation with 1% periodic acid followed by water rinsing and Schiff's reagent staining. Gels were imaged using the ChemiDoc MP Imaging System (Bio-Rad).

Immunoblotting—Separated proteins were electroblotted to nitrocellulose membranes for 60 min at 190 mA and 30 V (constant). Blots were blocked in 5% semi skimmed milk (Fluka) and probed with either an affinity-purified antibody fraction of mouse antiserum to a synthetic peptide of human cystatin-s corresponding to amino acid residues 21-141 (AF1296, R&D Systems) or an affinity-purified goat antibody raised against a peptide mapping at the C terminus of
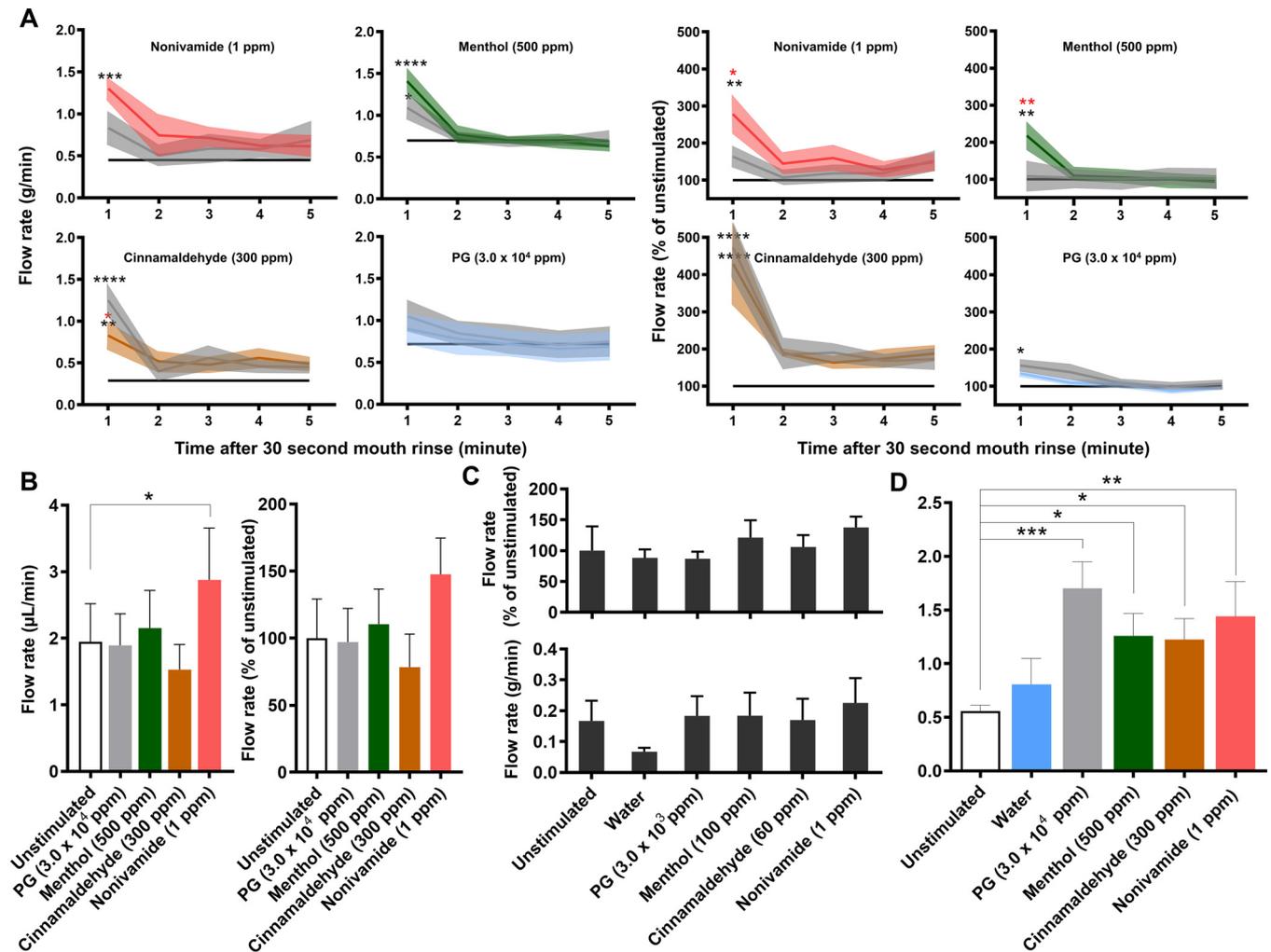
FIG. 1. **Effect of TRP channel agonists on salivary flow rates and protein output.** *A*, WMS flow rate after 30 s of mouth rinsing expressed as absolute values (*left*) and relative to the unstimulated flow rate (*right*) (*n* = 6). Solid colored lines indicate means and shaded areas indicate S.E. gray indicates vehicle control (PG) at concentration used for the TRP agonist mouth rinse. Black line indicates mean unstimulated WMS flow rate. The blue line in the PG plots indicate water. Black * indicates significance *versus* unstimulated and red * indicates significance *versus* PG. *B*, Lower labial minor salivary gland flow rate after 2 mins of mouth rinsing *(*Mean ± S.E.; *n* = 10). *C*, Parotid saliva flow rate during 2 mins of mouth rinsing (Mean ± S.E.; *n* = 8). *D*, WMS protein output after 30 s of mouth rinsing *(*Mean ± S.E.; *n* = 6).

human amylase (sc-12821, Santa Cruz). Binding was detected using a horseradish-peroxidase-labeled, affinity purified goat-ant-rabbit IgG (P0160, Agilent Dako) or rabbit-anti-mouse IgG (P0161, Agilent Dako) followed by Clarity Western ECL substrate detection system. Chemiluminescence was detected by ChemiDoc MP Imaging System (Bio-Rad). Molecular masses were determined by comparison with SeeBlue Plus2 standard proteins (Thermo Scientific).

Ethics—This study was approved by the King's College London Ethics Committee (BDM/12/13-54) and written informed consent was obtained from all study participants.

Statistical Analysis—Data were tested for normality using the Shapiro-Wilks normality test. 1-way ANOVA were used for determining statistically significant differences within the lower labial gland flow rates, parotid gland flow rates, protein output, cystatin S abundance data sets and, in the in-depth analysis, grouped WMS flow rate and protein output data sets. A 2-way ANOVA was used for determining statistically significant differences within the WMS flow rate data sets and, in the in-depth analysis, in the subject separated

WMS flow rate and protein output data sets. The above analyses were carried out using Prism 6 software (GraphPad). The following were used to denote statistically significant differences in the figures: **** = $p \leq 0.0001$, *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$.

RESULTS

TRP Agonists Stimulate Salivary Secretion—Significantly greater relative WMS flow rates were observed in response to the TRP agonist containing mouth rinses when compared with the UWMS flow rate (Fig. 1*A*). Furthermore, 1 ppm nonivamide and 500 ppm menthol mouth rinsing significantly increased relative mean WMS flow rates compared with PG mouth rinsing, which itself significantly increased WMS flow rates compared with UWMS. The reproducibility of WMS flow rates in response to menthol and nonivamide mouth
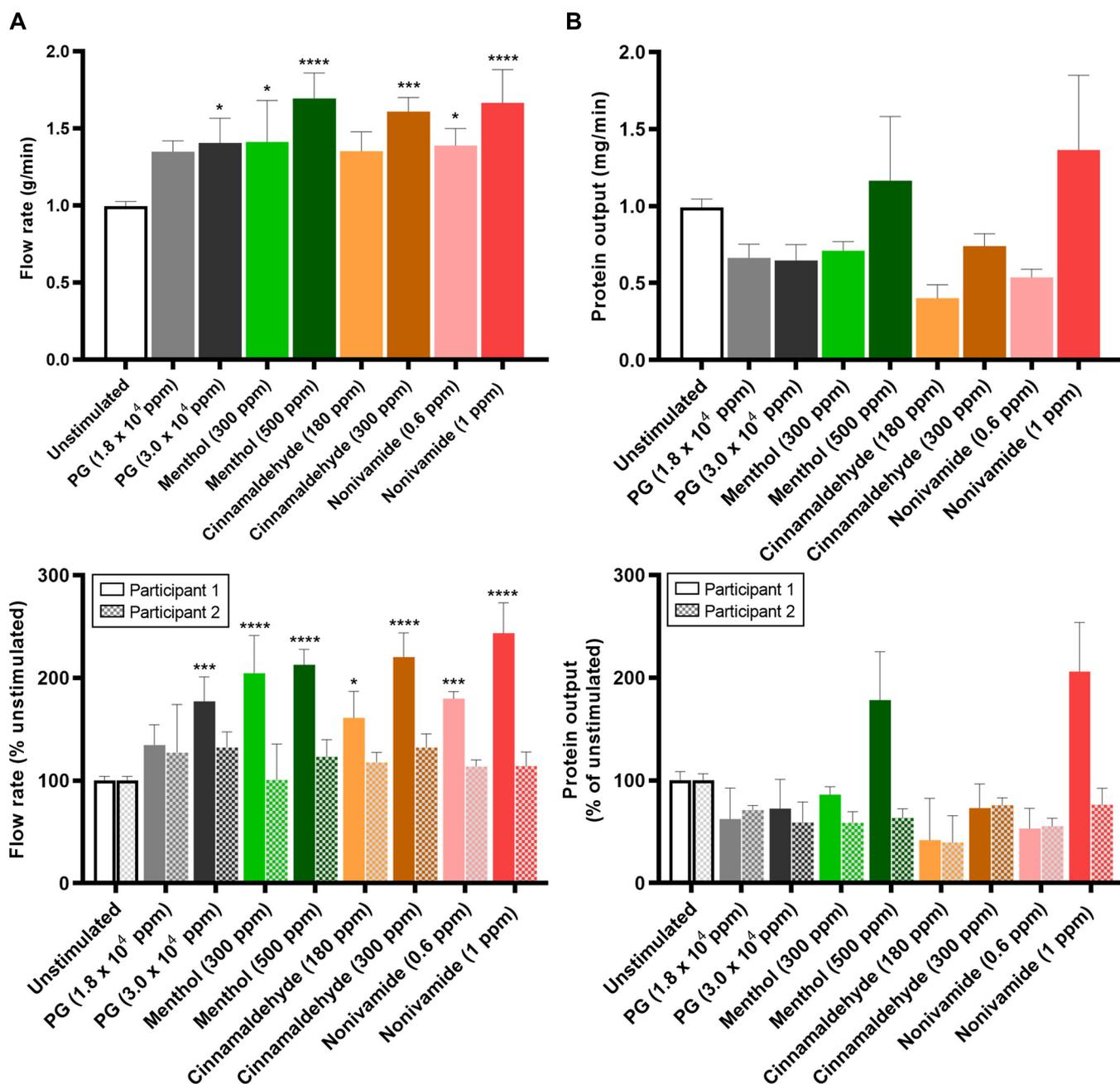
FIG. 2. **Reproducibility of the sialogogic properties of TRP channel mouth rinses.** *A*, WMS flow rates of unstimulated saliva and stimulated saliva during the first minute after mouth rinse stimulation (*top*) and participant separated values relative to the unstimulated flow rate on the day of sampling (*bottom*). *B*, WMS protein output of unstimulated saliva and post-mouth rinse salivas in the 2 mins after stimulation (*top*) and participant separated values relative to unstimulated protein output (*bottom*). All figures show mean ± S.E. Top figures: $n = 6$, unstimulated $n = 48$; Bottom figures: $n = 3$, unstimulated $n = 24$; *, **, *** and **** = P value from unstimulated $\leq$ 0.05, 0.01, 0.001 and 0.0001 respectively.

rinsing was demonstrated by repeating measurements with two of the participants (Fig. 2A). All the mouth rinses increased mean WMS flow rate compared with unstimulated WMS (UWMS) flow rate (1.0 g/min). The highest concentrations of the three TRP channel agonists stimulated the greatest flow rates; 1.70 ml/min with 500 ppm menthol, 1.61 g/min with 300 ppm cinnamaldehyde and 1.67 g/min with 1 ppm nonivamide (Fig. 2A (*top*)). When individ-

ual participants were considered, Fig. 2A (*bottom*), we found that only participant 1 showed significantly greater stimulated flow rates.

Nonivamide (1 ppm) mouth rinsing stimulated lower labial minor gland flow rate compared with the unstimulated flow rate (Fig. 1B) but no mouth rinse caused parotid gland flow rates to significantly differ from unstimulated or water stimulated flows (Fig. 1C).
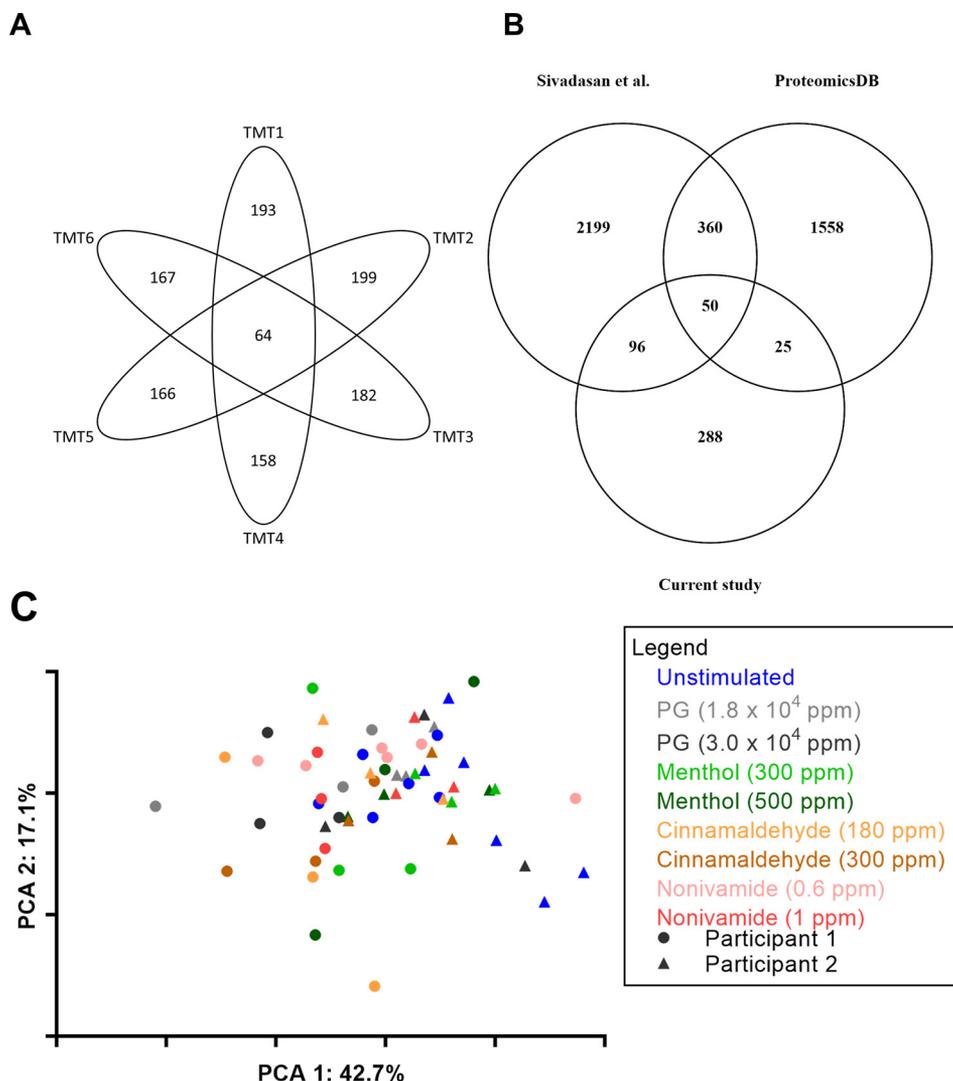
FIG. 3. **Proteomics overview.** *A,* Venn diagram showing total number of identified proteins in each TMT10plex (outer) and the number of proteins identified in all TMT10plexes (inner) for all samples in each TMT10plex. *B,* Venn diagram showing the unique and common proteins identified in the current study, from a reference database (ProteomicsDB) and a meta-analysis of the salivary proteome by Sivadasan *et al.* 2015. *C,* PCA plot showing the distribution of unstimulated pools and post-mouth rinse WMS sample after ComBat batch correction.

TRP agonist mouth rinsing, as well as PG, caused greater WMS protein output (Fig. 1*D*). These effects were shown to be less reproducible than the effects on flow rate (Fig. 2*B versus* 3*A*). Although mean output in response to 1 ppm nonivamide (1.36 mg/min) and 500 ppm menthol (1.17 mg/min) were greater than UWMS (0.99 mg/min), these increases were not significant and can be attributed to participant 1, who showed a significantly greater response than participant 2 (Fig. 2*D*).

Salivary Proteomics Overview—Overall 459 unique proteins were identified in saliva samples. The number of unique proteins identified in each of the 6 separate batches of samples varied from 199 to 158. Sixty four unique proteins were identified in all 6 sample batches (Fig. 3*A*). Two reference proteomes were used to compare the proteins identified in this study to those identified in the literature. In a meta-analysis

of proteins identified across six studies, Sivadasan *et al.* produced the largest publicly available "human salivary proteome," consisting of 3449 unique human proteins (44). A second reference proteome was obtained from ProteomeDB (https://www.proteomsdb.org/) which contained 1993 unique human proteins.

Our study identified 288 unique human proteins absent from both data sets and so, to the best of our knowledge, are novel findings for the salivary proteome (Fig. 3*B*). Greater confidence can be assigned to the 134 proteins that have a SwissProt annotation score of 5, relating to strong evidence of their existence *in vivo*, and of these, 12 were identified with at least one unique peptide across the batches, of which 9 had a relative abundance of less than 0.2%.

Sources of Variation in the Salivary Proteome—When all samples were labeled by participant and condition (Fig. 3*C*),
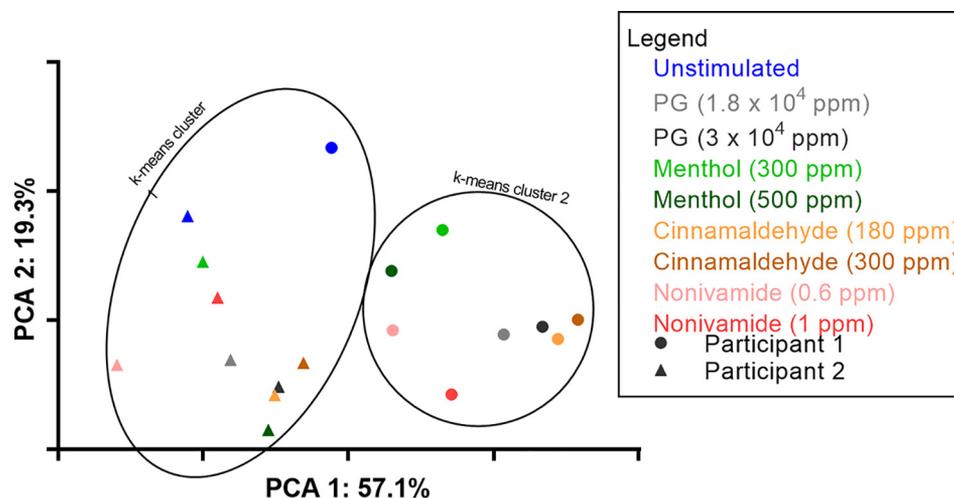
FIG. 4. **Identification of sources of variation in the salivary proteome.** A PCA plot showing the distribution of the geomean of each of the sample conditions with highlighted k-means clusters.

samples are discriminated by participant along the x axis (PCA1). Furthermore, if the geomean of the replicates of each condition are taken (Fig. 4) and k-means clustering (number of clusters having been determined by x-means) applied then 100% of participant 2 samples cluster together and 89% of participant 1 samples cluster together. All stimulated samples from participant 2 clustered separately from the unstimulated sample, reflecting that this subject was a responder. In contrast none of the stimulated samples from participant 1 clustered separately from unstimulated samples, reflecting that this subject was a nonresponder. Because the x axis represents the principal component responsible for the majority of the variation in the data set (57.1%), we conclude that the person the saliva comes is the major source of variation between WMS proteomes.

The geomeans of post-mouth rinse samples were separated by mouth rinse primarily on the y axis of Fig. 4, representing the principal component responsible for 19.3% of variation in the data set. For both participants, post-PG and cinnamaldehyde mouth rinse coordinates associated together, suggesting that the cinnamaldehyde mouth rinses were not causing additional variation in the WMS proteome than was already induced by the PG in the mouth rinse. However, post-nonivamide and menthol coordinates were separated from the PG coordinates suggesting these compounds were inducing proteome changes independently of PG (note the lower concentrations of PG in nonivamide and menthol mouth rinses compared with cinnamaldehyde (Table II).

Supplemental Fig. *B* shows the mean ($\pm$S.E.) variability of each post-mouth rinse sample to the unstimulated pool in both participants. Nonivamide caused changes in the WMS proteome in both participants, 1 ppm in participant 1 and 0.6 ppm in participant 2. Cinnamaldehyde (300 ppm) and to a lesser degree menthol (300 ppm) caused relatively large

changes in the WMS proteome of participant 1. Large variation was sometimes seen in the proteome response to the same mouth rinse in the same participant, as indicated by the large S.E. values, for example in participant 1–300 ppm menthol and participant 2–0.6 ppm nonivamide. In contrast, some mouth rinses cause very repeatable changes, for example 300 ppm menthol in participant 2 and 0.6 ppm nonivamide in participant 1.

Specific Protein Changes—Ten unique proteins were significantly regulated by TRP channel agonist stimulation (Table IV), five of which belong to the cystatin family. Salivary cystatins (S, SA or SN) were up-regulated in response to every mouth rinse with the greatest degree of up-regulation observed in response to nonivamide mouth rinses. The peptides assigned to each of these proteins (13, 10 and 17 to S, SA and SN respectively) were unique. Additionally, cystatin D was up-regulated at both concentrations of nonivamide and cystatin C was up-regulated after 1 ppm nonivamide mouth rinsing. Menthol at 500 ppm caused up-regulation in salivary cystatins to a greater extent than PG. Although salivary cystatins were up-regulated after cinnamaldehyde mouth rinsing, it was less than with PG mouth rinses despite the same concentration of PG being present in 1.8×10^4 ppm and 3.0×10^4 ppm PG to 180 ppm and 300 ppm cinnamaldehyde respectively. The finding that salivary cystatins are up-regulated by 1 ppm nonivamide mouth rinsing was supported by qualitative immunoprobing (Fig. 5). Statistically significant greater cystatin S was observed in WMS after 1 ppm nonivamide mouth rinsing (Fig. 5*C*).

Two other proteins were up-regulated in the data set, prolactin-inducible protein was up-regulated after both PG and cinnamaldehyde mouth rinsing whereas neutrophil defensin 1 (α-defensin) was up-regulated in response to PG (Table IV).

TABLE IV

WMS proteins regulated by TRP channel agonist mouth rinsing. Fold change in geomean (compared to unstimulated saliva) of WMS proteins after rinsing with TRP channel agonist or vehicle with significant regulation (p < 0.05) across both participants. Fold changes recognized as up- or downregulated are highlighted in bold and italic respectively. Blanks indicate that protein was present but not regulated. Additionally: the total number of peptides identified across all 6 batches is reported as well as the mean protein coverage across the six batches

| Protein ID	Protein Name	Total Peptides Identified (% of total)	Mean Protein Coverage (%)	PG		Cinnamaldehyde		Menthol		Nonivamide	
				1.8×10^4 Ppm	3.0×10^4 Ppm	180 ppm	300 ppm	300 ppm	500 ppm	0.6 ppm	1 ppm
P12273	Prolactin-inducible protein	258 (0.95)	13.58	**1.92**	**1.82**	**1.60**	**1.73**				
P59665	Neutrophil defensin 1	367 (1.35)	24.83	**1.62**	**1.57**						
P01034	Cystatin-C	205 (0.76)	40.41								**1.56**
P28325	Cystatin-D	202 (0.75)	31.80							**1.64**	**1.79**
P01036	Cystatin-S	1227 (4.53)	76.59		**1.57**	**1.59**	**1.61**		**1.66**	**1.81**	**1.72**
P09228	Cystatin-SA	326 (1.2)	38.89		**2.08**	**1.72**	**1.87**	**1.77**	**2.02**	**2.15**	**2.14**
P01037	Cystatin-SN	4024 (14.84)	66.55		**1.52**				**1.68**	**1.82**	**1.79**
P01860	Ig gamma-3 chain C region	74 (0.27)	14.15			*0.52*				*0.63*	*0.56*
Q9BWT7	CARD10	74 (0.27)	1.45			*0.66*	*0.66*				
P00558	Phosphoglycerate kinase 1	60 (0.22)	7.00			*0.43*					
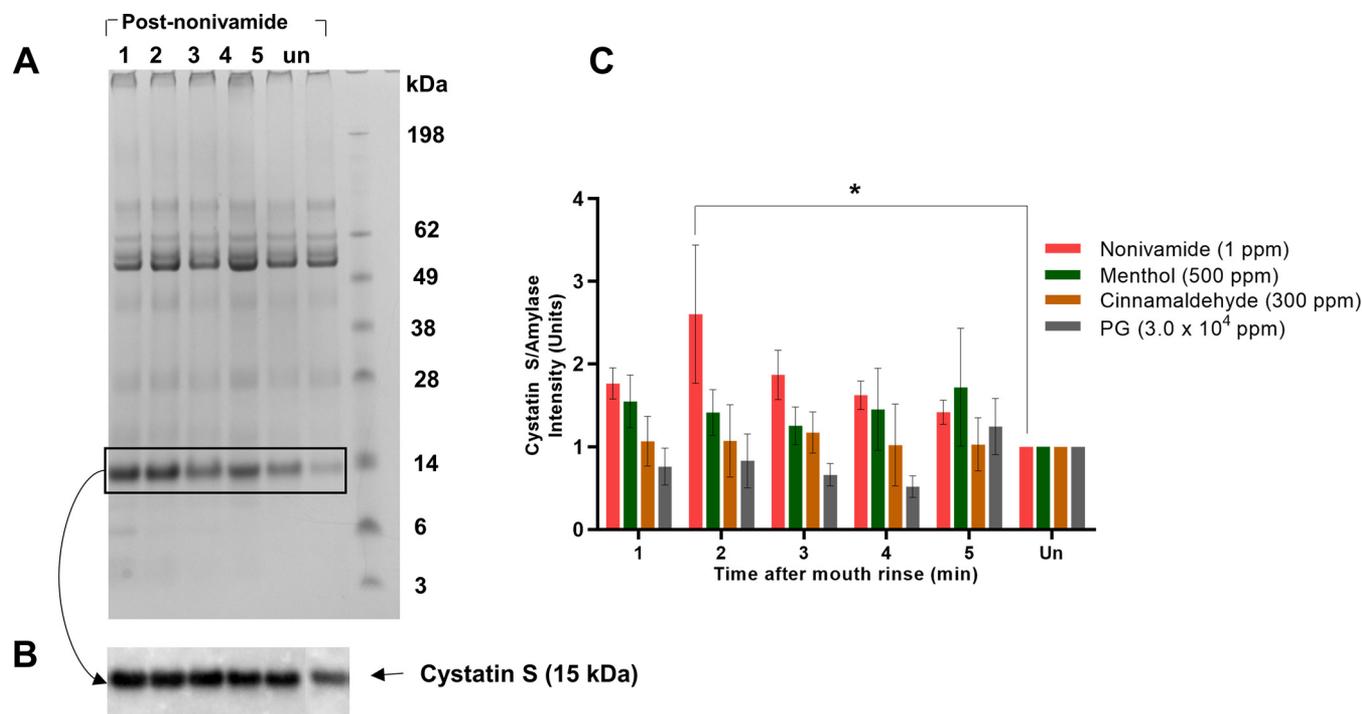
FIG. 5. **WMS cystatin S abundance after TRP channel agonist mouth rinsing.** *A,* An example of Coomassie blue and PAS stained salivary proteins separated by SDS-PAGE from one participant demonstrating how the cystatin S band intensities increase after nonivamide *B,* Western blot of the same samples as in a) identifying the protein band as cystatin S. (un: unstimulated, 1 - 5: 1 - 5 min after mouth rinse. *C,* Intensity of the cystatin S band on a western blot, relative to the amylase Western blot band intensity, in WMS collected after a 30 s TRP agonist mouth rinse normalized to unstimulated saliva (Mean±S.E.; *n* = 6).

Cinnamaldehyde (180 ppm) resulted in the down-regulation of IgG-3 chain C region, caspase recruitment domain-containing protein 10 (CARD10) (also down-regulated in 300 ppm cinnamaldehyde) and phosphoglycerate kinase 1 (PGK1). IgG-3 chain C region was also down-regulated in response to nonivamide.

DISCUSSION

In this study we have found that mouth rinsing with menthol or nonivamide increases WMS flow rate (Figs. 1 and 2). These observations expand on the current reports in the literature that TRPV1 agonists, such as piperine, nonivamide, capsaicin and 6-gingerol can stimulate salivary secretion because stimulation of salivary secretion by menthol has not previously been described. We have further found that nonivamide can stimulate minor gland secretion. Cinnamaldehyde mouth rinse did not evoke a salivary response even though it was perceived to be as intense or more intense than the menthol or nonivamide mouth rinses (supplemental Data C), which indicates that salivary responses are TRP agonist specific. The effect of a cinnamaldehyde mouth rinse was no greater than the vehicle PG but both were greater than unstimulated WMS (Fig. 1A). Nonivamide, menthol and PG increased outputs of total protein in saliva suggesting that the protein composition and properties of saliva might be altered. Cinnamaldehyde decreased protein secretion compared with the PG vehicle. This is likely because of cinnamaldehyde diminishing the sialogogic properties of PG through a reaction between the compounds rather than inhibiting the nerve mediated reflex PG induces as no inhibitory neurones exist (45). The source of increased protein secretion is presumably salivary gland exocytosis of protein storage granules but it may be that there are other contributions from within the oral cavity. In order to investigate further, quantitative changes in salivary protein composition we implemented a bottom-up MS pipeline new to salivary proteomics, which led to the identification of novel whole WMS proteome changes and specific protein changes in response to the TRP channel agonists studied. From PCA we identified that the largest source of variation in the salivary proteome was between subjects but that changes in the proteome were also caused by different mouth rinses (Fig. 4). Repeat analyses on subjects demonstrated that there was variation from day to day in response to some of the mouth rinses.

The MS pipeline applied in this study produced results that contribute to the salivary proteome literature, because it identified proteins in saliva that have not previously been reported (supplemental Table). This may be because of the novel application of IEF using OFFGEL electrophoresis with TMT labelled quantitative tandem MS LC–MS/MS to salivary proteomics but may also be the result of searching against updated databases or inter-personal differences in salivary composition, which has previously been observed to have a larger coefficient of variation than intra-personal variation (46). Three previous studies of WMS have used IEF in tandem MS (47–49), and a further study coupled it with mTRAQ quantification methodology (50). However, these studies did not couple IEF with isobaric labeling such as TMT. It could be that the novel methodology contributes to better identification of lower abundance proteins, or this could be a result of the experimental stochasticity in bottom-up MS approaches, the use of updated protein sequence database or differences in raw data analysis software. Despite being in lower abundance, the novel proteins are of sufficient length (median amino acid length being 897 and ranging from 97 to 7570) to produce detectable tryptic peptides. This suggests that the method is not just identifying small proteins with a high abundance but proteins of a range of sizes with relative abundances ranging from 3.2% of total peptides to $< 0.005\%$ (supplemental Table). A bottom-up approach was implemented with the intention to maximize the quantification of the salivary proteome. With 459 proteins quantified, the coverage was limited when compared with other TMT quantification studies with more state-of-the-art equipment. Furthermore, good proteome coverage that also represents the variety of gene products has been achieved in top-down and data independent acquisition proteomic studies and could be used to further investigate the diversity of the salivary proteome (51, 52).

The presence of some lower abundance proteins appeared to be influenced by mouth rinsing, for example CARD10 and phosphoglycerate kinase 1 (PGK1), which were 0.3 and 0.2% of total identified peptides respectively (Table IV). This is the first time CARD10 has been identified in WMS. Both CARD10 and PGK1 were down-regulated specifically in response to cinnamaldehyde mouth rinsing. Despite there being no previous reports of association between CARD10 and cinnamaldehyde, there have been previous reports of cinnamaldehyde inhibiting other caspase recruitment domain proteins in mice and subsequent anti-inflammatory effects (10). Similarly, there have been no previous reports of an association between cinnamaldehyde and PGK1. However, anti-angiogenesis properties of cinnamaldehyde and cinnamon extract have been previously reported (12–14). The observation of down-regulation of CARD10 and PGK1 could be preliminary evidence that the anti-inflammatory and bactericidal effects of cinnamaldehyde extend to short term mouth rinsing in the oral cavity.

Upregulation of cystatin S in the WMS secreted in response to nonivamide was detected by MS and western blotting (Fig. 5). Despite significant sequence homology between the salivary cystatins, the peptides assigned to S, SN and SA were unique to each protein. Furthermore, the antibody used in western blotting had a reasonable specificity for cystatin S, with 30 and 5% cross-reactivity to cystatins SN/SA or D/C respectively. To further increase the confidence in specificity, a top down approach could be used as demonstrated in the literature (53). Greater quantities of cystatin S in saliva could result in an improvement in mucosal adhesion, a property of saliva important in mouthfeel and xerostomia. Cystatin S has been shown to interact with oral mucosal surfaces and play a role in the formation of protein pellicles *in vitro* on hydrophobic surfaces that mimic the mucosa (54). Coupled with previous observations that the

rheological properties of saliva are modified by nonivamide (29, 55), mouth rinsing with nonivamide as a treatment for xerostomia warrants further study. Increased cystatin S expression may have other potential benefits for oral health. because of inhibition of cysteine protease activity, as indicated by significant enrichment of the "negative regulation of cysteine-type endopeptidase activity" GO. The up-regulation of the GO for cysteine protease inhibition mirrors the western blotting findings and work in the literature (56, 57). Cystatin S has been shown to inhibit proteolytic activity in the culture supernatant of *P. gingivalis* (58), a Gram-negative bacterial species that produces the gingipain class of cysteine proteases which are implicated in periodontal disease (59). Additionally, cystatin S, as well as prolactin-inducible protein, up-regulation could improve acceptance of bitter taste as indicated by the GO enrichment "detection of chemical stimulus involved in sensory perception of bitter taste" (60). This suggests that TRPV1 agonists could be used to promote the consumption of bitter foods, the reduced consumption of which has been implicated in the health, dietary intake and weight of "super tasters" (61).

This study is the first to demonstrate an acute salivary cystatin S response to TRPV1 agonists in humans (Fig. 5). A cystatin S-like protein response to capsaicin has been demonstrated in rats fed on a capsaicin-adulterated diet; the presence of a new protein in rat saliva was demonstrated and the protein found to have cystatin S-like properties such as inhibition of cysteine protease activity (57). In the rat increased cystatin S-like protein levels enhanced consumption of a capsaicin rich diet and it was hypothesized that this response may be triggered by irritation of the oral mucosa (56). Although these studies, along with the current study, both show increases in cystatin S and cystatin S-like proteins in saliva, the time scales over which the phenomenon occurs are significantly different. The current study shows the reversible increase within 2 mins of nonivamide mouth rinsing whereas in the studies in rat the increase was observed after 3 days of capsaicin-adulterated diet, suggesting different mechanisms are responsible. The increase in cystatin S levels in WMS in the current study must be because of the release of preformed protein as it takes 30 min for newly synthesized protein containing vesicles to pass from the rough endoplasmic reticulum to the condensing vacuoles in secretory cells (62).

The identification of proteins regulated across all mouth rinses alongside proteins only regulated in response to one mouth rinse suggests, in agreement with the total protein secretion data, that there are different mechanisms responsible for the regulation of proteins in WMS. Furthermore, some of the proteins are known to be produced by the salivary glands whereas others are nonsalivary proteins. The up-regulation of salivary cystatins (S, SN and SA) may reflect a preferential stimulation of the submandibular/sublingual glands, the primary producers of salivary cystatins (63). Cystatin S

regulation may be influenced by direct effects of the agonists on minor glands, as lower labial gland flow rates were greater after 1 ppm nonivamide mouth rinsing (Fig. 1*B*) and they have been demonstrated to express cystatin S and other salivary proteins (64). Menthol, cinnamaldehyde and nonivamide are highly lipophilic compounds, having partition coefficient values (an indicator of lipophilicity; higher values imply greater lipophilicity) of 3, 1.9, and 4.2 respectively. Comparatively, pilocarpine, a drug that has previously been used to directly stimulate minor salivary glands (65), has a partition coefficient value of 1.1 (66). Higher lipophilicity suggests that these TRP channel agonists would have a greater permeability in the oral epithelium and lamina propria than pilocarpine, which would enhance direct activation of TRP channels expressed in minor glands.

The significantly greater WMS flow rates observed in the proteomics study (Fig. 2*A* response to the TRP agonists. There is a precedence in sensory science for responders/nonresponders, such as in the case of the detection of the bitter compound PROP which is associated with the expression of the TAS2R28 bitter receptor gene (67). Although the comparison seems to be limited by the fact that participants in the current study do have a sensory perception of the TRP agonists, the mechanism for salivary secretion in response to TRP agonist detection is yet to be elucidated and unknown genetic factors could be responsible for the prevalence of salivary nonresponders to TRP agonists despite a sensory perception. A breakdown of the data set shown in Fig. 1*A* reveals that only 2 of the 19 participants given a TRP containing mouth rinse did not exhibit an increase in WMS flow rate (as defined by a flow rate 150% that of unstimulated flow rate). This suggests that the prevalence of nonresponders in the population is lower than the 50% suggested in the proteomics study.

In summary this study provides the first evidence for stimulation of salivary secretion by a nonTRPV1 TRP channel agonist. Increased minor gland secretion may be a direct action of the TRP agonists on submucosal salivary glands alongside nerve-mediated mechanisms. Furthermore, novel changes in the proteome of the saliva secreted in response to the TRPV1 agonist nonivamide were identified by MS and supported by western blotting. These findings suggest that TRP channel agonists can be explored as potential candidates for altering salivary secretion, particularly in subjects with xerostomia and reduced levels of saliva.

DATA AVAILABILITY

The PD 1.4 protein search file result containing accession numbers, percentage protein coverage, number of distinct peptides and quantification measurements can be found in supplemental Tables 1–6. The raw-files, msf-files and PD1.4 search files (protein and peptide) have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD017232.

Abbreviations—The abbreviations used are: CARD10, caspase recruitment domain-containing protein 10; DTT, dithiothreitol; LC-MS/MS, liquid chromatography - tandem mass spectrometry; GO, Gene Ontology; IEF, isoelectric focusing; LDS, lithium dodecyl sulfate; MS, mass spectrometry; PG, propylene glycol; PGK1, phosphoglycerate kinase 1; TCEP, tris (2-CarboxyEthyl) phosphine; TEAB, triethylammonium bicarbonate; TMT, tandem mass tag; TRP, transient receptor potential; TRPA1, transient receptor potential cation channel, subfamily A, member 1; TRPM8, transient receptor potential cation channel subfamily M member 8; TRPV1, transient receptor potential cation channel subfamily V member 1; WMS, whole mouth saliva; UWMS, unstimulated whole mouth saliva.

REFERENCES

1. Hand, A. R., and Frank, M. E. (2014) *Fundamentals of oral histology and physiology*. Ames, Iowa: John Wiley & Sons Inc p. p.
2. Kido, M. A., Muroya, H., Yamaza, T., Terada, Y., and Tanaka, T. (2003) Vanilloid receptor expression in the rat tongue and palate. *J. Dent. Res.* **82,** 393–397
3. Ishida, Y., Ugawa, S., Ueda, T., Murakami, S., and Shimada, S. (2002) Vanilloid receptor subtype-1 (VR1) is specifically localized to taste papillae. *Brain Res. Mol. Brain Res.* **107,** 17–22
4. Wang, B., Danjo, A., Kajiya, H., Okabe, K., and Kido, M. A. (2011) Oral epithelial cells are activated via TRP channels. *J. Dent. Res.* **90,** 163–167
5. Lyall, V., Heck, G. L., Vinnikova, A. K., Ghosh, S., Phan, T.-H. T., Alam, R. I., Russell, O. F., Malik, S. A., Bigbee, J. W., and DeSimone, J. A. (2004) The mammalian amiloride-insensitive non-specific salt taste receptor is a vanilloid receptor-1 variant. *J. Physiol.* **558,** 147–159
6. Smith, K. R., Treesukosol, Y., Paedae, A. B., Contreras, R. J., and Spector, A. C. (2012) Contribution of the TRPV1 channel to salt taste quality in mice as assessed by conditioned taste aversion generalization and chorda tympani nerve responses. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **303,** R1195–R205
7. Bán, A., Marincsák, R., Bíró, T., Perkecz, A., Gömöri, E., Sándor, K., Tóth, I. B., Bánvölgyi, A., Szolcsányi, J., and Pintér, E. (2010) Upregulation of transient receptor potential vanilloid type-1 receptor expression in oral Lichen Planus. *Neuroimmunomodulat* **17,** 103–108
8. Bandell, M., Story, G. M., Hwang, S. W., Viswanath, V., Eid, S. R., Petrus, M. J., Earley, T. J., and Patapoutian, A. (2004) Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron* **41,** 849–857
9. Lee, S. H., Lee, S. Y., Son, D. J., Lee, H., Yoo, H. S., Song, S., Oh, K. W., Han, D. C., Kwon, B. M., and Hong, J. T. (2005) Inhibitory effect of 2′-hydroxy-cinnamaldehyde on nitric oxide production through inhibition of NF-κB activation in RAW 264.7 cells. *Biochem. Pharmacol.* **69,** 791–799
10. Lee, S. C., Wang, S. Y., Li, C. C., and Liu, C. T. (2018) Anti-inflammatory effect of cinnamaldehyde and linalool from the leaf essential oil of Cinnamomum osmophloeum Kanehira in endotoxin-induced mice. *J Food Drug Anal* **26,** 211–220
11. Hancı, D., Altun, H., Çetinkaya, E. A., Muluk, N. B., Cengiz, B. P., and Cingi, C. (2016) Cinnamaldehyde is an effective anti-inflammatory agent for treatment of allergic rhinitis in a rat model. *Int. J. Pediatr. Otorhinolaryngol.* **84,** 81–87
12. Eisenberg, D. M., Harris, E. S. J., Littlefield, B. A., Cao, S., Craycroft, J. A., Scholten, R., Bayliss, P., Fu, Y., Wang, W., Qiao, Y., Zhao, Z., Chen, H., Liu, Y., Kaptchuk, T., Hahn, W. C., Wang, X., Roberts, T., Shamu, C. E., and Clardy, J. (2011) Developing a library of authenticated Traditional Chinese Medicinal (TCM) plants for systematic biological evaluation - Rationale, methods and preliminary results from a Sino-American collaboration. *Fitoterapia* **82,** 17–33
13. Kwon, B.-M., Lee, S.-H., Cho, Y.-K., Bok, S.-H., So, S.-H., Youn, M.-R., and Chang, S.-I. (1997) Synthesis and biological activity of cinnamaldehydes as angiogenesis inhibitors. *Bioorg Med Chem Lett* **7,** 2473–2476
14. Kwon, H.-K., Jeon, W. K., Hwang, J.-S., Lee, C.-G., So, J.-S., Park, J.-A., Ko, B. S., and Im, S.-H. (2009) Cinnamon extract suppresses tumor progression by modulating angiogenesis and the effector function of CD8(+) T cells. *Cancer Lett.* **278,** 174–182
15. Wu, S.-J., Ng, L.-T., and Lin, C.-C. (2005) Cinnamaldehyde-induced apoptosis in human PLC/PRF/5 cells through activation of the proapoptotic Bcl-2 family proteins and MAPK pathway. *Life Sci.* **77,** 938–951
16. Ka, H., Park, H.-J., Jung, H.-J., Choi, J.-W., Cho, K.-S., Ha, J., and Lee, K.-T. (2003) Cinnamaldehyde induces apoptosis by ROS-mediated mitochondrial permeability transition in human promyelocytic leukemia HL-60 cells. *Cancer Lett.* **196,** 143–152
17. Park, K.-R., Nam, D., Yun, H.-M., Lee, S.-G., Jang, H.-J., Sethi, G., Cho, S. K., and Ahn, K. S. (2011) β-Caryophyllene oxide inhibits growth and induces apoptosis through the suppression of PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation. *Cancer Lett.* **312,** 178–188
18. Ng, L.-T., and Wu, S.-J. (2011) Antiproliferative activity of Cinnamomum cassia constituents and effects of pifithrin-alpha on their apoptotic signaling pathways in Hep G2 cells. *Evidence-Based Complementary and Alternative Medicine* **2011,** 1–6
19. Peier, A. M., Moqrich, A., Hergarden, A. C., Reeve, A. J., Andersson, D. A., Story, G. M., Earley, T. J., Dragoni, I., McIntyre, P., Bevan, S., and Patapoutian, A. (2002) A TRP channel that senses cold stimuli and menthol. *Cell* **108,** 705–715
20. Haas, J. S., Whipple, R. E., Grant, P. M., Andresen, B. D., Volpe, A. M., and Pelkey, G. E. (1997) Chemical and elemental comparison of two formulations of oleoresin capsicum. *Sci. Justice* **37,** 15–24
21. Watanabe, S., and Dawes, C. (1988) The effects of different foods and concentrations of citric-acid on the flow-rate of whole saliva in man. *Arch. Oral Biol.* **33,** 1–5
22. Chauncey, H. H., Shannon, I. L., and Feller, R. P. (1963) Effect of acid solutions on human gustatory chemoreceptors as determined by parotid gland secretion rate. *Proc. Soc. Exp. Biol. Med.* **112,** 917–923
23. Hodson, N. A., and Linden, R. W. A. (2006) The effect of monosodium glutamate on parotid salivary flow in comparison to the response to representatives of the other four basic tastes. *Physiology., and Behavior* **89,** 711–717
24. Stolle, T., Grondinger, F., Dunkel, A., Meng, C., Médard, G., Kuster, B., and Hofmann, T. (2017) Salivary proteome patterns affecting human salt taste sensitivity. *J. Agric. Food Chem.* **65,** 9275–9286
25. Nasrawi, C. W., and Pangborn, R. M. (1990) Temporal gustatory and salivary responses to capsaicin upon repeated stimulation. *Physiol. Behav.* **47,** 611–615
26. Lawless, H., Rozin, P., and Shenker, J. (1985) Effects of oral capsaicin on gustatory, olfactory and irritant sensations and flavor identification in humans who regularly or rarely consume chili pepper. *Chem. Senses* **10,** 579–589
27. Dunér-Engström, M., Fredholm, B. B., Larsson, O., Lundberg, J. M., and Saria, A. (1986) Autonomic mechanisms underlying capsaicin induces oral sensations and salivation in man. *J. Physiol. (Lond)* **373,** 87–96
28. Kono, Y., Kubota, A., Taira, M., Katsuyama, N., and Sugimoto, K. (2018) Effects of oral stimulation with capsaicin on salivary secretion and neural activities in the autonomic system and the brain. *J. Dent. Sci.* **13,** 116–123
29. Gardner, A., So, P.-W., and Carpenter, G. (2020) Endogenous salivary citrate is associated with enhanced rheological properties following oral capsaicin stimulation. *Exp. Physiol.* **105,** 96–107

30. Thomson, W. M., Poulton, R., Broadbent, J. M., and Al-Kubaisy, S. (2006) Xerostomia and medications among 32-year-olds. *Acta Odontol. Scand*. **64,** 249–254

31. Carpenter, G. H. (2015) *Dry Mouth*. 1 ed: Springer-Verlag Berlin Heidelberg

32. Lussi, A., Schlueter, N., Rakhmatullina, E., and Ganss, C. (2011) Dental erosion–an overview with emphasis on chemical and histopathological aspects. *Caries Res*. **45 Suppl 1,** 2–12

33. Dawes, C. (1987) Physiological factors affecting salivary flow-rate, oral sugar clearance, and the sensation of dry mouth in man. *J. Dent. Res*. **66,** 648–653

34. Ship, J. A., Fox, P. C., and Baum, B. J. (1991) How much saliva is enough - normal function defined. *J. Am. Dent. Assoc*. **122,** 63–69

35. Pramanik, R., Osailan, S. M., Challacombe, S. J., Urquhart, D., and Proctor, G. B. (2010) Protein and mucin retention on oral mucosal surfaces in dry mouth patients. *Eur. J. Oral Sci*. **118,** 245–253

36. Carpenter, G. H. (2015) Artificial salivas: why are they not more useful? In: Carpenter GH, editor. *Dry Mouth: A Clinical Guide on Causes, Effects and Treatments*. 1 ed: Springer-Verlag Berlin Heidelberg

37. Lee, V. M., and Linden, R. W. (1992) An olfactory-submandibular salivary reflex in humans. *Exp. Physiol*. **77,** 221–224

38. Lee, V. M., and Linden, R. W. A. (1992) The effect of odours on stimulated parotid salivary flow in humans. *Physiol. Behav*. **52,** 1121–1125

39. Mackie, D. A., and Pangborn, R. M. (1990) Mastication and its influence on human salivary flow and alpha-amylase secretion. *Physiol. Behav*. **47,** 593–595

40. Neyraud, E., Sayd, T., Morzel, M., and Dransfield, E. (2006) Proteomic analysis of human whole and parotid salivas following stimulation by different tastes. *J. Proteome Res*. **5,** 2474–2480

41. Bader, M., Dunkel, A., Wenning, M., Kohler, B., Medard, G., Del Castillo, E., Gholami, A., Kuster, B., Scherer, S., and Hofmann, T. (2018) Dynamic proteome alteration and functional modulation of human saliva induced by dietary chemosensory stimuli. *J. Agric. Food Chem*. **66,** 5621–5634

42. Bader, M., Stolle, T., Jennerwein, M., Hauck, J., Sahin, B., and Hofmann, T. (2018) Chemosensate-induced modulation of the salivary proteome and metabolome alters the sensory perception of salt taste and odor-active thiols. *J. Agric. Food Chem*. **66,** 7740–7749

43. Johnson, W. E., Li, C., and Rabinovic, A. (2007) Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* **8,** 118–127

44. Sivadasan, P., Gupta, M. K., Sathe, G. J., Balakrishnan, L., Palit, P., Gowda, H., Suresh, A., Kuriakose, M. A., and Sirdeshmukh, R. (2015) Human salivary proteome - a resource of potential biomarkers for oral cancer. *J. Proteomics*. **127,** 89–95

45. Garrett, J. R., Ekstrom, J., and Anderson, L. C. (1999) Neural mechanisms of salivary gland secretion. In: Linden RWA, editor. *Frontiers of oral biology:* Karger p. 26-43

46. Jehmlich, N., Dinh, K. H. D., Gesell-Salazar, M., Hammer, E., Steil, L., Dhople, V. M., Schurmann, C., Holtfreter, B., Kocher, T., and Völker, U. (2013) Quantitative analysis of the intra- and inter-subject variability of the whole salivary proteome. *J. Periodont. Res*. **48,** 392–403

47. Bencharit, S., Altarawneh, S. K., Baxter, S. S., Carlson, J., Ross, G. F., Border, M. B., Mack, C. R., Byrd, W. C., Dibble, C. F., Barros, S., Loewy, Z., and Offenbacher, S. (2012) Elucidating role of salivary proteins in denture stomatitis using a proteomic approach. *Mol. Biosyst*. **8,** 3216–3223

48. Bandhakavi, S., Stone, M. D., Onsongo, G., Van Riper, S. K., and Griffin, T. J. (2009) A dynamic range compression and three-dimensional peptide fractionation analysis platform expands proteome coverage and the diagnostic potential of whole saliva. *J. Proteome Res*. **8,** 5590–5600

49. Cargile, B. J., Bundy, J. L., Freeman, T. W., and Stephenson, J. L. (2004) Gel based isoelectric focusing of peptides and the utility of isoelectric point in protein identification. *J. Proteome Res*. **3,** 112–119

50. Bandhakavi, S., Van Riper, S. K., Tawfik, P. N., Stone, M. D., Haddad, T., Rhodus, N. L., Carlis, J. V., and Griffin, T. J. (2011) Hexapeptide libraries for enhanced protein PTM identification and relative abundance profiling in whole human saliva. *J. Proteome Res*. **10,** 1052–1061

51. Bruderer, R., Bernhardt, O. M., Gandhi, T., Xuan, Y., Sondermann, J., Schmidt, M., Gomez-Varela, D., and Reiter, L. (2017) Optimization of experimental parameters in data-independent mass spectrometry significantly increases depth and reproducibility of results. *Mol. Cell. Proteomics* **16,** 2296–2309

52. Smith, L. M., Kelleher, N. L., Linial, M., Goodlett, D., Langridge-Smith, P., Ah Goo, Y., *et al*. Consortium for Top Down Proteomics, (2013) Proteoform: a single term describing protein complexity. *Nat. Methods* **10,** 186–187

53. Manconi, B., Liori, B., Cabras, T., Vincenzoni, F., Iavarone, F., Castagnola, M., Messana, I., and Olianas, A. (2017) Salivary cystatins: exploring new post-translational modifications and polymorphisms by top-down high-resolution mass spectrometry. *J. Proteome Res*. **16,** 4196–4207

54. Yakubov, G. E., Macakova, L., Wilson, S., Windust, J. H. C., and Stokes, J. R. (2015) Aqueous lubrication by fractionated salivary proteins: Synergistic interaction of mucin polymer brush with low molecular weight macromolecules. *Tribol. Int*. **89,** 34–45

55. Houghton, J. W., Hans, J., Pesaro, M., Ley, J. P., Carpenter, G. H., and Proctor, G. (2017) Sensory effects of transient receptor potential channel agonists on whole mouth saliva extensional rheology. *J. Texture Stud*. **48,** 313–317

56. Katsukawa, H., Shang, Y., Nakashima, K., Yang, K. H., Ohashi, R., Sugita, D., Mishima, K., Nakata, M., Ninomiya, Y., and Sugimura, T. (2002) Salivary cystatins influence ingestion of capsaicin-containing diets in the rat. *Life Sci*. **71,** 457–467

57. Katsukawa, H., and Ninomiya, Y. (1999) Capsaicin induces cystatin S-like substances in submandibular saliva of the rat. *J. Dent. Res*. **78,** 1609–1616

58. Blankenvoorde, M. F. J., Henskens, Y. M. C., vantHof, W., Veerman, E. C. I., and Amerongen, A. V. N. (1996) Inhibition of the growth end cysteine proteinase activity of Porphyromonas gingivalis by human salivary cystatin S and chicken cystatin. *Biol. Chem*. **377,** 847–850

59. How, K. Y., Song, K. P., and Chan, K. G. (2016) Porphyromonas gingivalis: An Overview of Periodontopathic Pathogen below the Gum Line. *Front. Microbiol*. **7,** 53

60. Morzel, M., Chabanet, C., Schwartz, C., Lucchi, G., Ducoroy, P., and Nicklaus, S. (2014) Salivary protein profiles are linked to bitter taste acceptance in infants. *Eur. J. Pediatr*. **173,** 575–582

61. Chamoun, E., Mutch, D. M., Allen-Vercoe, E., Buchholz, A. C., Duncan, A. M., Spriet, L. L., Haines, J., and Ma, D. W. L, Guelph Family Health Study, (2018) A review of the associations between single nucleotide polymorphisms in taste receptors, eating behaviors, and health. *Crit. Rev. Food Sci. Nutr*. **58,** 194–207

62. Palade, G. (1975) Intracellular aspects of the process of protein synthesis. *Science* **189,** 347–358

63. Baron, A. C., DeCarlo, A. A., and Featherstone, J. D. B. (1999) Functional aspects of the human salivary cystatins in the oral environment. *Oral Dis*. **5,** 234–240

64. Siqueira, W. L., Salih, E., Wan, D. L., Helmerhorst, E. J., and Oppenheim, F. G. (2008) Proteome of human minor salivary gland secretion. *J. Dent. Res*. **87,** 445–450

65. Taweechaisupapong, S., Pesee, M., Aromdee, C., Laopaiboon, M., and Khunkitti, W. (2006) Efficacy of pilocarpine lozenge for post-radiation xerostomia in patients with head and neck cancer. *Aust. Dental J*. **51,** 333–337

66. Kim, S., Thiessen, P. A., Bolton, E. E., Chen, J., Fu, G., Gindulyte, A., Han, L., He, J., He, S., Shoemaker, B. A., Wang, J., Yu, B., Zhang, J., and Bryant, S. H. (2016) PubChem Substance and Compound databases. *Nucleic Acids Res*. **44,** D1202–D13

67. Duffy, V. B., Davidson, A. C., Kidd, J. R., Kidd, K. K., Speed, W. C., Pakstis, A. J., *et al*. (2004) Bitter Receptor Gene (TAS2R38), 6-n-Propylthiouracil (PROP) Bitterness and Alcohol Intake. *Alcoholism: Clin. Exp. Res*. **28,** 1629–1637