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Proteome data set of human gingival crevicular fluid from healthy periodontium sites by multidimensional protein separation and mass spectrometry

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Background and Objective: Gingival crevicular fluid has been of major interest for many decades as a valuable body fluid that may serve as a source of biomarkers for both periodontal and systemic diseases. Owing to its very small sample size, submicroliter volumes, identification of its protein composition by classical biochemical methods has been limited. The advent of highly sensitive mass spectrometric technology has permitted large-scale identification of protein components of many biological samples. This technology has been employed to identify the protein composition of gingival crevicular fluid from inflamed and periodontal sites. In this report, we present a proteome data set of gingival crevicular fluid from healthy periodontium sites.

Material and Methods: A combination of a periopaper collection method with application of multidimensional protein separation and mass spectrometric technology led to a large-scale documentation of the proteome of gingival crevicular fluid from healthy periodontium sites.

Results: The approaches used have culminated in identification of 199 proteins in gingival crevicular fluid of periodontally healthy sites. The present gingival crevicular fluid proteome from healthy sites was compared and contrasted with those proteomes of gingival crevicular fluid from inflamed and periodontal sites, as well as serum. The cross-correlation of the gingival crevicular fluid and plasma proteomes permitted dissociation of the 199 identified gingival crevicular fluid proteins into 105 proteins (57%) that can be identified in plasma and 94 proteins (43%) that are distinct and unique to the gingival crevicular fluid microenvironment. Such analysis also revealed distinctions in protein functional categories between serum proteins and those specific to the gingival crevicular fluid microenvironment.

Conclusion: Firstly, the data presented herein provide the proteome of gingival crevicular fluid from periodontally healthy sites through establishment of innovative analytical approaches for effective analysis of gingival crevicular fluid from periopapers both at the level of complete elusion and with removal of abundant albumin, which restricts identification of low-abundant proteins. Secondly, it adds significantly to the knowledge of gingival crevicular fluid composition and highlights new groups of proteins specific to the gingival crevicular fluid microenvironment.

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Gingival crevicular fluid has been of major interest because of its unique capacity to reflect in its protein composition the alterations that occur during the transition from gingival health to periodontal disease. Gingival crevicular fluid is an inflammatory transudate or exudate originating from the gingival plexus of blood vessels in the gingival corium, subjacent to the epithelium lining the dentogingival space. Although various methods for gingival crevicular fluid collection have been described, the most commonly used is the collection with paper strips placed in the sulcus (1). It can be easily and noninvasively collected, and its constituents have the capacity to reflect both local and systemically derived factors and macromolecules. Owing to these properties and the volume needed for analysis being much less than for serum or urine, for several decades gingival crevicular fluid was considered as a potentially valuable body fluid that may serve as an important source of biomarkers for both periodontal and systemic diseases (1–11). In this respect, numerous gingival crevicular fluid-derived inflammatory factors, such as cytokines, proteins, proteinases, phosphatases and local tissue degradation products, can be found in gingival crevicular fluid and have been evaluated as possible diagnostic markers for periodontitis using traditional methods, such as ELISA (2,9,10,12–14). It is clear that in-depth and more comprehensive documentation of the gingival crevicular fluid proteome is of major interest and desired.

The major protein components of body fluids such as urine, serum/plasma (15,16), whole saliva, parotid secretion and submandibular/sublingual secretions (17–21) have been established by classical biochemistry approaches. In more recent times, the advent of mass spectrometry (MS) led to a large-scale proteome documentation of these body fluids, plasma (22), urine (23,24) and, within the dental field, the proteome of whole saliva (25,26), parotid secretion (27–29), minor gland secretion (25,28–31), acquired enamel pellicle (31,32) and the large-scale phosphoproteome of whole saliva (33,34). Despite these technological advances, the large-scale proteome analysis and extent of data for gingival crevicular fluid components still remain significantly limited. One of the major limitations to the establishment of more extensive knowledge of the protein composition of gingival crevicular fluid, using both classical biochemical methods and MS technology, has been the availability of only very small quantities of gingival crevicular fluid (~0.2–0.5 μL per site) for periodontally healthy individuals.

Recently, a study has been reported describing an attempt to assess the MS-based protein composition of gingival crevicular fluid from inflamed gingival sites of patients who had history of periodontal disease and were in the maintenance phase of treatment. The reason for using gingival crevicular fluid from such patients to carry out that study was suggested to be that the volume of gingival crevicular fluid from healthy individuals was too low to permit proteome analysis even with sensitive MS technology (13). This is not strictly true, because ~80 μg/μL and 0.2–0.5 μL per site is not a small amount of protein, in particular if one is to use the contemporary highly sensitive MS technology. Therefore, it is not the small volume of gingival crevicular fluid from periodontally healthy sites that may be limiting, but rather the collection method that becomes a limiting factor. The method of gingival crevicular fluid collection in that study used microcapillary glass tubes, and perhaps that was one of the limitations for not being able to collect sufficient gingival crevicular fluid fluid from healthy individuals. The use of inflamed/periodontal sites, even though the disease was in the maintenance phase, would provide more material because the pocket size/depth and gingival crevicular fluid volume increases in such patients. It should be noted, however, that the small quantity of gingival crevicular fluid material may not be the only limitation for the high sensitivity of the more contemporary MS technology. There are other limitations, such as dynamic protein range and presence of highly abundant proteins. Such limitations are clearly applicable to gingival crevicular fluid, which contains abundant serum-derived proteins. These become highy significant when contributions of serum change from 30% serum for gingival crevicular fluid from periodontally healthy individuals to 70% serum for those with inflamed sites or periodontal disease (35–37). In this case, the well-known dynamic protein range becomes a hindrance, whereby the presence of highly abundant serum proteins, such as albumin, constituting ~50% of the total serum proteins, and immunoglobulins, can and do restrict the identification of lower level proteins of both systemic and local origin (37–39). This is because the levels of albumin, immunoglobulins and other abundant serum proteins will most probably limit the scope of identified proteins using MS technology. These are well-known major issues and difficulties encountered in the establishment of the serum/plasma proteome by MS technology (38). Furthermore, the serum-related contributions become accentuated during gingivitis and periodontitis, with increased contributions from serum as well as additional proteins from the local inflammatory response. A recent study reported the use of periopapers for gingival crevicular fluid collection from a 21 d experimentally induced human gingivitis model to investigate alterations in protein levels as a function of inflammation (40).

To date, a large-scale proteome of gingival crevicular fluid from healthy periodontium sites still remains undefined, despite the fact that this knowledge is fundamentally central in establishing foundations for saliva-based diagnostic markers for systemic and periodontal diseases. In the present work, we report a large-scale proteome of gingival crevicular fluid from 'gingivally healthy sites', identifying 199 distinct proteins with high confidence using a periopaper collection method and 'multidimensional' mass spectrometric approach.

Material and methods

Gingival crevicular fluid collection

Gingival crevicular fluid collection protocols were approved by the institutional review board of Boston University Medical Center, and informed

consent was obtained from each subject participating in this study. Inclusion criteria included overall systemic and oral health, no current or recent medications, nonsmoking and not pregnant or lactating. Gingival crevicular fluid samples were collected from the healthy gingival sulcus of the second and third molar teeth. The patient evaluation and gingival crevicular fluid collections were all performed by the same trained and calibrated clinical dentist. In this study, the healthy 'periodontium site' was defined by the following clinical criteria: gingival index scored as 0, pocket depth $\leq$ 3 mm, clinical attachment level $\leq$ 1.5 mm and no bleeding on probing. These criteria were applied to specific sites from which collections were made, as well as the full mouth. Teeth were first cleaned with a water jet, gently air-dried and isolated with cotton rolls placed in the mesio-buccal sulcus. Commercially available collection periopaper strips were inserted into the orifice of the sulcus of periodontally healthy subjects for 30 s. While gingival crevicular fluid collection with periopaper from periodontally healthy sites very rarely showed signs of blood contamination, when this occurred those samples were discarded from analysis. The gingival crevicular fluid volume collected with the strips was determined after collection using a Periotron 8000 (Oraflow, Plainview, NY, USA), which was calibrated, with a standard curve constructed using a range of volumes of water. Gingival crevicular fluid was collected from nine gingivally healthy subjects, with four sites from each subject and at least two different quadrants. The nine subjects were four men and five women aged between 22 and 33 years, with a mean age of 28.5 $\pm$ 3.7 years. A total of 36 strips, each containing $\sim$0.3–0.6 μL of gingival crevicular fluid, were used and processed for MS analysis as described below.

Processing and analysis of gingival crevicular fluid proteins by nano-flow liquid chromatography electrospray ionization tandem mass spectrometry

In one set of experiments, the proteins were eluted from nine combined strips (pool 1) and another set of eight combined strips (pool 2) with 200 μL of 50 mM NH_4HCO_3, pH 8.0, followed by centrifugation at 7000 *g* for 5 min to collect the eluate. This was repeated three times, and the eluates were combined for each pool separately, trypsin digested followed by nano-flow liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) analysis. In a second set of experiments, to increase the number of proteins identified and maximize the identification of the low-level proteins in gingival crevicular fluid, the highly abundant serum albumin was removed by 'SwellGel Blue' (Pierce Co., Rockford, IL, USA) treatment. In this experiment, the proteins were eluted from 10 strips with 200 μL of 50 mM NH_4HCO_3, pH 8.0, followed by centrifugation to collect the eluate. This was repeated three times and the eluates combined. The effectiveness of the 'SwellGel Blue' albumin removal was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an aliquot of the eluate, which indicated almost complete removal of the albumin as defined by Coomassie blue staining. However, because of the sensitivity of the MS technology there were detectable traces of albumin present or its peptide fragments, which cannot be removed easily. The eluate after albumin removal, the SwellGel Blue and the periopaper strips were all subjected individually to trypsin digestion in the presence of 50 mM NH_4HCO_3, pH $\sim$8.0, and each sample subjected to LC-ESI-MS/MS analysis. The pooling of gingival crevicular fluid samples was used in order to reduce the site-specific variability in the constituents of gingival crevicular fluid (13).

SDS-PAGE of gingival crevicular fluid proteins and LC-ESI-MS/MS

To further characterize the gingival crevicular fluid proteome, another set of nine periopaper strips from nine subjects (one strip from each) were initially subjected to SDS-PAGE, and then different molecular weight sections were separately trypsin digested in the gel, followed by LC-ESI-MS/MS analysis.

Gingival crevicular fluid paper strips were placed in the PAGE (NuPage 12% Bis-Tris Gel 1.0 mm $\times$ 10 well) wells (one per well) and subjected to SDS-PAGE for extraction and separation of proteins by molecular weight. Electrophoresis was carried out at 120 V until the dye front reached to the bottom of the gel. After electrophoresis, the gel was stained with Coomassie blue and destained with a 40% methanol, 10% acetic acid solution. The destaining gel was sectioned into five different molecular weight ranges by excising these regions with a sharp straight-edged razor. The sections comprised cut 1 (above 75 kDa), cut 2 (75–50 kDa), cut 3 (below 50–35 kDa), cut 4 (below 25 to above 10 kDa) and cut 5 (10 kDa and below), as shown in Fig. 2. Each section was then cut into smaller pieces (1–2 mm) and placed in an Eppendorf tube. Sodium dodecyl sulfate and Coomassie blue stain were removed by treatment with buffer 1 (50 mM NH_4HCO_3, pH 8.0) and buffer 2 (25 mM NH_4HCO_3, pH 8.0, and 50% CH_3CN) alternately. This step was repeated three times. After the last buffer treatment, the gel pieces were suspended in buffer 1 containing trypsin (0.5 μg of trypsin per 25 μL of buffer) and the samples were incubated in Eppendorf tubes for 24 h at 37°C. Following in-gel digestion, buffer containing trypsin and the released peptides were removed and placed in an Eppendorf tube. The gel pieces were further extracted by addition of buffer 1 and buffer 2 alternately, with brief agitation (5 min) at each stage, and the extraction buffers were pooled. This process was repeated three times. The pooled extractions from each SDS-PAGE specific molecular weight range were then freeze dried and cleaned using a C-18 reverse-phase MacroSpin column (The Nest Group, Inc., Southborough, MA, USA) for removal of SDS and salts from samples prior to LC-ESI-MS/MS analysis.

Nano-flow liquid chromatography and electrospray ionization tandem mass spectrometric analysis

Liquid chromatography and electrospray ionization tandem mass spectrometric

analyses were carried out using an LTQ-linear ion trap mass spectrometer (Thermo Electron, San Jose, CA, USA). Samples were suspended in 97.4% H_2O, 2.5% CH_3CN and 0.1% formic acid, and LC-ESI-MS/MS analyses were carried out using an online autosampler (Micro AS; Thermo Finnigan, San Jose, CA, USA) with auto-injections of 3 μL onto an in-line fused silica microcapillary column (75 μm × 10 cm), packed in house with C_{18} resin (Micron Bioresource, Inc., Auburn, CA, USA) at a flow rate of 250 nL/min. Peptides were separated by a 55 min elution comprising a multistep linear gradient using solvent A (H_2O, 2.5% CH_3CN and 0.1% formic acid) and solvent B (CH_3CN and 0.1% formic acid). The gradient steps were from 100% solvent A to 8% solvent B in 5 min, to 15% solvent B in 10 min, to 25% solvent B in 10 min, to 50% solvent B in 20 min, and to 95% solvent B in 10 min using a Surveyor MS Pump Plus (Thermo Finnigan). The eluted peptides were directly nano-electrosprayed, and the MS/MS data were generated using data-dependent acquisition with an MS survey scan range between 390 and 2000 m/z. This data-dependent acquisition begins with the LC separation, which generates a total ion chromatogram in a survey scan, followed by selection of specific ions for collision-induced dissociation (CID; MS/MS) in descending order of signal intensity. Each survey scan (MS) was followed by automated sequential selection of the five most abundant peptides for CID, at 35% normalized collision energy, with a dynamic exclusion time of 20 s of the previously selected ions. This process was continuously alternated between MS survey scan and five tandem MS throughout the nano-LC chromatography.

Database search and protein/peptide identification

All MS/MS spectra from LC-ESI-MS/MS were searched against the human database, Uniprot (UNIVERSAL PROTEIN RESOURCE, version 9.0), which combines the data from SWISS-PROT (version 51), TREMBL (version 36) and PIR using BIOWORKS 3.3.1 (Thermo Electron, San Jose, CA, USA) software and the SEQUEST search engine (41). The data were searched against 241,242 entries. To determine the 'false-positive rate', the data were searched against a concatenated human sequence database containing both the forward and the reverse sequence version. The false-positive rate was calculated by using the number of matches to the reverse database multiplied by two and divided by the total number of matches (forward plus reverse), as described by Peng *et al.* (42). The DTA generation was with a precursor-ion tolerance of 1.5 atomic mass unit (amu), a fragment ion tolerance of 1.0 amu, and automated calculated charged states of +1, +2 and +3. The searches were carried out using partial trypsin specification and two miscleavages. DTA is a mass spectral file produced during SEQUEST analysis; it contains MS data for single or grouped scans. The use of partial trypsin searches was to avoid the exclusion of any peptides generated by the oral proteolytic activity known to occur in gingival crevicular fluid (27,43,44). The full tryptic and half-tryptic peptides were used to create the gingival crevicular fluid proteome list in Table 1. The database search results were filtered using the following criteria: peptide sequence coverage, ΔCn ≥ 0.1; probability ≤ 0.1; for fully tryptic peptides, cross-correlation, XCorr ≥ 1.6, 1.8 and 3.5 for charged state, Z = +1, +2 and +3; and for partial tryptic peptides, XCorr ≥ 1.8, 2.1 and 3.75 for Z = +1, +2 and +3.

In addition to the search parameters and criteria used, the identified peptide sequences were stringently evaluated and assessed manually by examining each of the identified peptide MS/MS data for the quality and the confidence through the b and y ion fragment series.

Protein annotations

The identified proteins were classified and assigned by molecular function, biological process and cellular component using the following three web-based applications: the Babelomics database http://babelomics.bioinfo.cipf.es/index.html; the AmiGO database (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi advanced_query = yes); and the Swiss protein database (http://ca.expasy.org/).

ELISA for human albumin

The human albumin ELISA Kit from Bethy Laboratories, Inc. (Montgomery, TX, USA) was used for identification and quantitation of human albumin in gingival crevicular fluid essentially as described in the manufacturer's protocol. One hundred microliters of each albumin standard or appropriately diluted sample were added to the corresponding wells of the ready-to-use precoated plate, followed by incubation for 1 h at room temperature. The plate was washed four times with wash buffer, and buffer was removed. Then 100 μL of detection antibody was added to each well followed by incubation for 1 h at room temperature. The plate was washed four times with wash buffer, and buffer was removed. This step was followed by addition of 100 μL strepavidin-conjugated horseradish peroxidase to each well and incubation for 30 min at room temperature. The plate was washed four times with wash buffer, followed by addition of 100 μL of tetramethylbenzidine to each well and incubation in the dark for 30 min at room temperature. The reaction was stopped by the addition of diluted sulfuric acid, and the absorbance was measured at 450 nm. The data were plotted, and the human albumin concentrations in the samples were determined from the standard curve.

Results and Discussion

In recent times, there has been significant interest in whole saliva and major parotid secretions, with a view to establishing their global protein composition, namely the proteome. This was predominantly fuelled by the advances made in MS and the concept that such information will aid development of noninvasive oral and systemic diagnostic biomarkers (26–29,45). These extensive studies have

Table 1. Gingival crevicular fluid proteome, 199 proteins, documented for healthy periodondium sites

Accession number	Protein name	Number of peptides of each protein identified	Number of times each protein identified
Apoptosis and signal transduction			
[S]P31946	14-3-3 protein beta/alpha[e]	2	2
[S]P31947	14-3-3 protein sigma[abcde]	7	7
[S]P63104	14-3-3 protein zeta/delta[abce]	6	7
[S]P12814	Alpha-actinin-1[ce]	2	2
O43707	Alpha-actinin-4[ce]	2	2
[S]P02647	Apolipoprotein A-I[abcde]	13	8
[S]P02652	Apolipoprotein A-II[bce]	3	4
[S]P04114	Apolipoprotein B-100[abd]	3	5
[S]P02649	Apolipoprotein E[bd]	4	3
[S]O14791	Apolipoprotein-L1[bd]	2	3
P54259	Atrophin-1[b]	2	1
[S]P23528	Cofilin-1[be]	3	4
P13569	Cystic fibrosis transmembrane conductance regulator[a]	2	1
[S]P47929	Galectin-7[be]	2	3
P07900	Heat shock protein HSP 90-alpha[de]	2	2
Q5VST9	Obscurin[a]	3	2
Q8IV61	Ras guanyl-releasing protein 3[c]	2	1
Q13114	TNF receptor-associated factor 3[d]	2	1
Q7Z3T8	Zinc finger FYVE domain-containing protein 16[bc]	2	2
Cytoskeleton structural proteins			
[S]P60709	Actin, cytoplasmic 1[abcde]	7	7
[S]P61158	Actin-related protein 3[d]	3	1
P17661	Desmin[be]	2	4
[S]P15924	Desmoplakin[e]	2	3
[S]P02751	Fibronectin[d]	2	3
[S]P21333	Filamin-A[a]	2	1
[S]P06396	Gelsolin[bcde]	8	7
P78385	Keratin type II cuticular Hb3[e]	2	1
O76013	Keratin, type I cuticular Ha6[b]	2	2
[S]P02533	Keratin, type I cytoskeletal 14[b]	7	7
[S]P08779	Keratin, type I cytoskeletal 16[b]	5	6
[S]P08727	Keratin, type I cytoskeletal 19[b]	3	2
[S]P35527	Keratin, type I cytoskeletal 9[c]	3	11
P04264	Keratin, type II cytoskeletal 1[acd]	8	15
[S]Q7Z794	Keratin, type II cytoskeletal 1b[be]	2	6
[S]P35908	Keratin, type II cytoskeletal 2 epidermal[ab]	4	12
Q01546	Keratin, type II cytoskeletal 2 oral[e]	3	4
P12035	Keratin, type II cytoskeletal 3[e]	2	3
P19013	Keratin, type II cytoskeletal 4[b]	2	5
[S]P13647	Keratin, type II cytoskeletal 5[ab]	17	12
[S]P02538	Keratin, type II cytoskeletal 6A[abc]	22	10
[S]P04259	Keratin, type II cytoskeletal 6B[b]	5	5
Q14CN4	Keratin, type II cytoskeletal 72[d]	2	9
Q86Y46	Keratin, type II cytoskeletal 73[e]	2	3
Q9UPN3	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5[ce]	3	2
P11137	Microtubule-associated protein 2[e]	2	10
[S]P26038	Moesin[bde]	2	3
P41219	Peripherin[b]	2	3
O15020	Spectrin beta chain, brain 2[e]	2	1
[S]Q9Y490	Talin-1[e]	3	3
[S]P08670	Vimentin[be]	3	3
Extracellular structural and adhesion proteins			
[S]P01019	Angiotensinogen[bc]	2	2
P02461	Collagen alpha-1(III) chain[ab]	2	1
Q02388	Collagen alpha-1(VII) chain[ab]	2	2

Table 1. (Continued)

Accession number	Protein name	Number of peptides of each protein identified	Number of times each protein identified
Q99715	Collagen alpha-1(XII) chain[ab]	4	3
[S]Q14993	Collagen alpha-1(XIX) chain[b]	3	1
P08123	Collagen alpha-2(I) chain[ab]	2	3
Q8N613	Cornulin[be]	4	5
O60469	Down syndrome cell adhesion molecule[a]	2	1
[S]P02671	Fibrinogen alpha chain[bcde]	10	4
[S]P02675	Fibrinogen beta chain[abd]	9	6
[S]P02679	Fibrinogen gamma chain[b]	10	4
P13645	Keratin, type I cytoskeletal 10[a]	4	9
[S]P13646	Keratin, type I cytoskeletal 13[a]	20	10
Q8WXI7	Mucin-16[e]	4	2
Q5BJF6	Outer dense fiber protein 2[e]	2	1
Q9BYM8	RanBP-type and C3HC4-type zinc finger-containing protein 1[be]	2	2
Q8IWN7	Retinitis pigmentosa 1-like1 protein[a]	2	1
[S]P02768	Serum albumin[abcde]	14	15
Q9UBC9	Small proline-rich protein 3[be]	7	6
[S]Q4LDE5	Sushi, von Willebrand factor type A[a]	3	4
Q9P2K2	Thioredoxin domain-containing protein 16[d]	4	9
Q9BQ70	Transcription factor 25[a]	2	1
P13611	Versican core protein[b]	2	1
[S]P04004	Vitronectin[bd]	5	3
Hydrolytic enzymes			
P49189	4-trimethylaminobutyraldehyde dehydrogenase[d]	2	2
P52209	6-phosphogluconate dehydrogenase, decarboxylating[de]	3	3
[S]P06733	Alpha-enolase[bcde]	8	7
[S]Q9NSC7	Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1[b]	2	1
Q6GMR7	Amidase domain containing protein[c]	3	1
P08311	Cathepsin G[b]	3	3
[S]P00450	Ceruloplasmin[abc]	7	5
[S]P04075	Fructose-bisphosphate aldolase A[de]	4	3
[S]P04406	Glyceraldehyde-3-phosphate dehydrogenase[de]	7	8
[S]P00738	Haptoglobin[bc]	2	7
Q6ZRS2	Helicase SRCAP[a]	2	3
Q8TEK3	Histone-lysine N-methyltransferase, H3 lysine-79 specific[bd]	4	3
P08246	Leukocyte elastase[b]	2	5
[S]P00338	L-lactate dehydrogenase A[e]	2	3
P43490	Nicotinamide phosphoribosyltransferase[b]	2	2
[S]P00558	Phosphoglycerate kinase 1[d]	2	1
[S]P18669	Phosphoglycerate mutase 1[bcde]	3	9
[S]P14618	Pyruvate kinase isozyme M1/M2[bde]	11	6
P55072	Transitional endoplasmic reticulum ATPase[a]	2	1
P29401	Transketolase[ce]	4	4
Q7Z410	Transmembrane protease, serine 9[d]	2	1
[S]P60174	Triosephosphate isomerase[e]	2	1

Table 1. (Continued)

Accession number	Protein name	Number of peptides of each protein identified	Number of times each protein identified
Inflammatory and immune response			
[S]P04083	Annexin A1[abde]	10	11
P06727	Apolipoprotein A-IV[b]	2	2
P17213	Bactericidal permeability-increasing protein[b]	2	1
[S]P10909	Clusterin[abde]	4	6
[S]P01024	Complement C3[abcde]	27	9
[S]P0C0L4	Complement C4-A[d]	7	2
[S]P00751	Complement factor B[bd]	4	3
[S]P31146	Coronin-1A[e]	2	2
[S]P01876	Ig alpha-1 chain C region[bcde]	2	6
[S]P01857	Ig gamma-1 chain C region[abcde]	10	14
[S]P01859	Ig gamma-2 chain C region[a]	6	7
[S]P01861	Ig gamma-4 chain C region[e]	2	4
[S]P01742	Ig heavy chain V-I region EU[d]	2	1
[S]P01834	Ig kappa chain C region[abcde]	4	11
[S]P01871	Ig mu chain C region[b]	2	2
[S]P02788	Lactotransferrin[a]	2	5
[S]P61626	Lysozyme C[e]	2	7
P05164	Myeloperoxidase[e]	2	1
[S]P05109	Protein S100-A8[a]	3	3
[S]P06702	Protein S100-A9[a]	2	2
[S]P52566	Rho GDP-dissociation inhibitor 2[e]	2	2
Intracellular protein/nucleotide binding			
P62736	Actin, aortic smooth muscle[abcde]	15	13
Q562R1	Actin-like protein 2[d]	2	5
Q3V5L5	Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase B[b]	2	3
Q6S8J3	ANKRD26-like family C member 1A[a]	11	11
Q9P2D7	Dynein heavy chain 1, axonemal[a]	2	3
[S]P68104	Elongation factor 1-alpha 1[be]	5	3
P15311	Ezrin[bd]	2	2
[S]P04792	Heat shock protein beta-1[bde]	6	9
[S]P69905	Hemoglobin subunit alpha[abcd]	2	8
[S]P68871	Hemoglobin subunit beta[abcd]	7	6
[S]P02042	Hemoglobin subunit delta[abcd]	2	1
[S]P02790	Hemopexin[abd]	7	3
[S]P16403	Histone H1.2[e]	2	1
P0C0S8	Histone H2A type 1[be]	3	7
Q96A08	Histone H2B type 1-A[e]	2	1
P33778	Histone H2B type 1-B[be]	2	5
P68431	Histone H3.1[abe]	2	7
Q16695	Histone H3.1t[b]	3	1
[S]P62805	Histone H4[abcde]	6	10
Q5JU85	IQ motif and Sec7 domain-containing protein 2[d]	2	1
Q96JM7	Lethal (3) malignant brain tumor-like 3 protein[b]	2	2
[S]P40925	Malate dehydrogenase[d]	2	1
Q6P0Q8	Microtubule-associated serine/threonine-protein kinase 2[b]	2	1
O14950	Myosin regulatory light chain MRLC2[b]	2	1
P35580	Myosin-10[e]	2	1
[S]P35579	Myosin-9[bde]	6	5
Q14686	Nuclear receptor coactivator 6[b]	2	1
P19338	Nucleolin[d]	2	1

Table 1. (Continued)

Accession number	Protein name	Number of peptides of each protein identified	Number of times each protein identified
P12755	Ski oncogene[d]	2	1
Q9P2P6	StAR-related lipid transfer protein 9[bc]	2	2
Q92922	SWI/SNF complex subunit SMARCC1[a]	2	2
Q86TI0	TBC1 domain family member 1[e]	2	1
Q8TBP0	TBC1 domain family member 16[d]	2	1
Q99973	Telomerase protein component 1[d]	2	1
Q9BVT8	Transmembrane and ubiquitin-like domain-containing protein 1[a]	2	2
Q71U36	Tubulin alpha-1A chain[e]	3	6
Q13748	Tubulin alpha-3C/D chain[e]	3	2
[S]P68366	Tubulin alpha-4A chain[bde]	3	4
[S]Q13885	Tubulin beta- 2A chain[e]	3	4
O43309	Zinc finger and SCAN domain-containing protein 12[b]	2	1
Protease/enzyme inhibitors			
[S]P01011	Alpha-1-antichymotrypsin[d]	2	1
[S]P01009	Alpha-1-antitrypsin[a]	4	2
[S]P01023	Alpha-2-macroglobulin[acde]	10	5
[S]P07355	Annexin A2[bde]	5	4
P12429	Annexin[e]	2	4
[S]P04080	Cystatin-B[a]	2	4
[S]P05546	Heparin cofactor 2[d]	2	1
[S]P04196	Histidine-rich glycoprotein[b]	2	
[S]P19827	Inter-alpha-trypsin inhibitor heavy chain H1[d]	6	1
[S]P19823	Inter-alpha-trypsin inhibitor heavy chain H2[d]	3	1
[S]Q14624	Inter-alpha-trypsin inhibitor heavy chain H4[e]	2	1
[S]P30740	Leukocyte elastase inhibitor[a]	2	1
[S]P30086	Phosphatidylethanolamine-binding protein 1[d]	2	1
Proteins falling into various other categories			
[S]P11021	78 kDa glucose-regulated protein[e]	2	1
Q8WWM7	Ataxin-2-like protein[d]	2	1
Q9BXY5	Calcyphosin-2[d]	2	1
[S]P27482	Calmodulin-like protein 3[be]	2	5
Q8IV04	Carabin[d]	2	1
Q7Z7A1	Centriolin[b]	3	1
P35663	Cylicin-1[b]	2	1
Q08477	Cytochrome P450 4F3[b]	2	1
P06737	Glycogen phosphorylase[de]	2	2
[S]P34931	Heat shock 70 kDa protein 1L[de]	2	5
P08238	Heat shock protein HSP 90-beta[bde]	2	1
Q9UHV7	Mediator of RNA polymerase II transcription subunit 13[bc]	3	2
[S]P62937	Peptidyl-prolyl cis-trans isomerase A[bde]	3	3
[S]P13796	Plastin-2[e]	2	4
Q12809	Potassium voltage-gated channel subfamily H member 2[c]	2	1
[S]P07737	Profilin-1[e]	3	6
[S]P07237	Protein disulfide-isomerase[e]	2	1
[S]P06703	Protein S100-A6[e]	2	2
P46940	Ras GTPase-activating-like protein[d]	3	1
P21817	Ryanodine receptor 1[e]	2	2

Table 1. (Continued)

Accession number	Protein name	Number of peptides of each protein identified	Number of times each protein identified
[S]P02787	Serotransferrin[a]	2	6
[S]P27169	Serum paraoxonase/arylesterase 1[b]	2	4
Q99536	Synaptic vesicle membrane protein VAT-1[b]	2	1
[S]Q8WZ42	Titin[abd]	47	10
[S]P02766	Transthyretin[a]	3	2
[S]P02774	Vitamin D-binding protein[c]	2	1
Q96KN7	X-linked retinitis pigmentosa GTPase regulator[b]	2	1
Q9H2Y7	Zinc finger protein 106 homolog[e]	2	1
[S]Q96DA0	Zymogen granule protein 16 homolog B[b]	3	5

The proteins have been grouped into seven different categories based on their known biological function(s). The superscript capital 'S' on the left of accession numbers denotes proteins that can also be identified in the plasma proteome (22) by MS analysis. The small superscript letters on the right of protein names denote the following: [a] proteins identified by electroelution using SDS-PAGE, dissected sections, in gel digested and analysed by LC-MS/MS; [b] proteins remaining on the periopaper after elution with ammonium bicarbonate; [c] proteins remaining on the SwellGel blue; [d] gingival crevicular fluid sample after albumin removal of proteins eluted from the SwellGel blue; and [e] direct analysis of the proteins eluted with ammonium bicarbonate 50 mM (pH 8.0) without any fractionation step or albumin removal.

been carried out using whole saliva or parotid secretions from healthy individuals, with no systemic or periodontal disease, in order to establish the baseline proteome in health, which can then be used for comparison with diseased states in order to discover diagnostic biomarkers. Another oral cavity-specific fluid is gingival crevicular fluid, which has a special protein composition, in that even in healthy periodondium the microenvironment of gingival crevicular fluid contains local proteins, such as cytokines, extracellular matrix components and degradation products, as well as serum-derived proteins. To date, the proteome of gingival crevicular fluid from periodontally healthy individuals by large-scale MS technology remains in its infancy. In-depth understanding of gingival crevicular fluid composition from periodontally healthy sites is a prerequisite as a baseline before disease states can be evaluated.

The present study identified and documented a proteome data set of gingival crevicular fluid from 'periodontally healthy sites' by multidimensional protein separation and tandem MS technology. Such an approach, in combination with the use of periopaper for sample collection, led to identification of 199 distinct proteins in gingival crevicular fluid, none of which was related to salivary secretion proteins (Table 1). This approach overcomes some of the protein 'dynamic range' limitations frequently encountered in large-scale proteome analysis by MS technology (38). The present study demonstrated that with multiple refined techniques it is possible to identify a proteome data set of gingival crevicular fluid from individuals with healthy periodontium. The gingival crevicular fluid proteome reported in Table 1 was constructed using only proteins identified by two or more peptides, which is the accepted criterion for general proteomic studies. Using the filtering criteria chosen, the results were associated with a false-positive rate of $< 2\%$. Figure 1 illustrates the multiple sample preparation steps that were undertaken to establish the present gingival crevicular fluid proteome data set. These steps of digesting what remained on the periopaper after elution and what adhered to SwellGel Blue with albumin provided an interesting set of results, which clearly indicated that this approach provided specific enrichment and sample simplification. There were 99 proteins identified when the periopapers themselves with residual proteins were subjected to trypsin digestion and the collected peptides analysed by MS. This indicated that some proteins have strong adsorption to these filters and they can be digested on the paper and peptides eluted for analysis. When the SwellGel Blue itself was subjected to trypsin digestion and MS analysis, 37 proteins were identified, indicating that in addition to albumin there were several proteins that were specifically adsorbed strongly to this gel or as complexes with albumin. After the albumin removal step, the eluate showed 71 proteins identified. Figure 2 shows the distribution of these proteins and their occurrence as common and uncommon identified proteins in each of these samples. Overall, these analyses led to the identification of 142 distinct proteins in this set. These data prompted us to incorporate an additional separation step based on relative molecular mass, using SDS-PAGE and analysing individual specific relative molecular mass sections containing different molecular weight proteins (Fig. 3). This approach provided identification of 54 proteins, of which 30 were common, with the direct method and after albumin removal, with an additional 24 new distinct proteins identified. A third set of data was obtained by simple elution of proteins from periopaper with

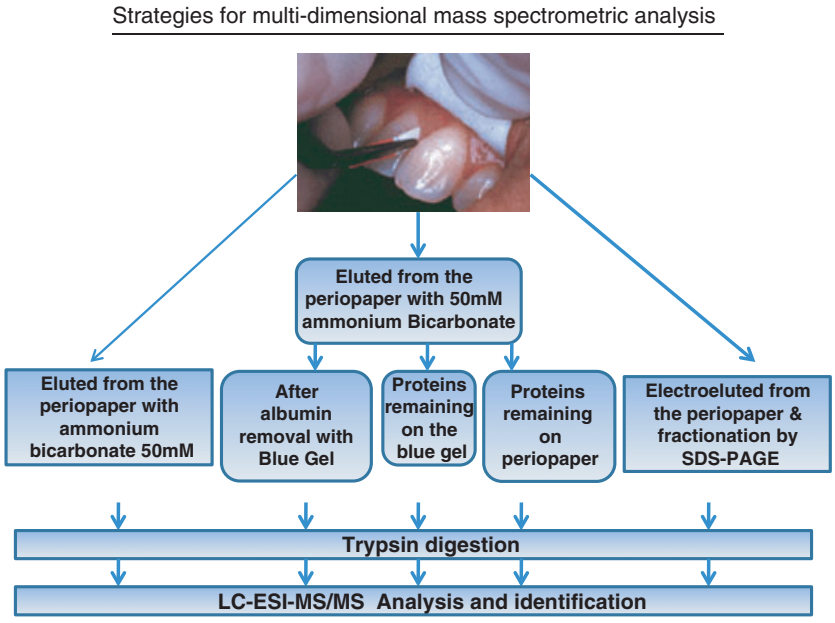

Fig. 1. Schematic representation of the multiple fractionation/separation techniques used and different processing of the gingival crevicular fluid periopaper samples for multidimensional mass spectrometric proteome analysis.

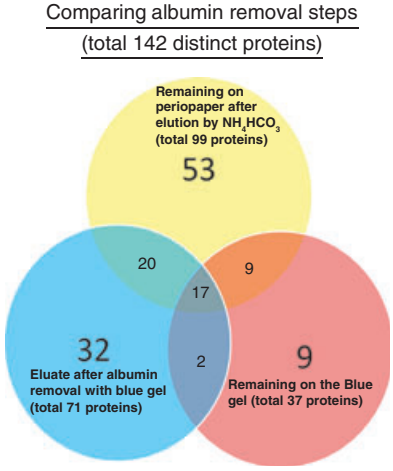

Fig. 2. Venn diagram summarizing the distinct proteins identified in the gingival crevicular fluid of healthy sites during albumin removal steps. Note the common and uncommon proteins identified in each of the samples, which culminated in 142 distinct proteins.

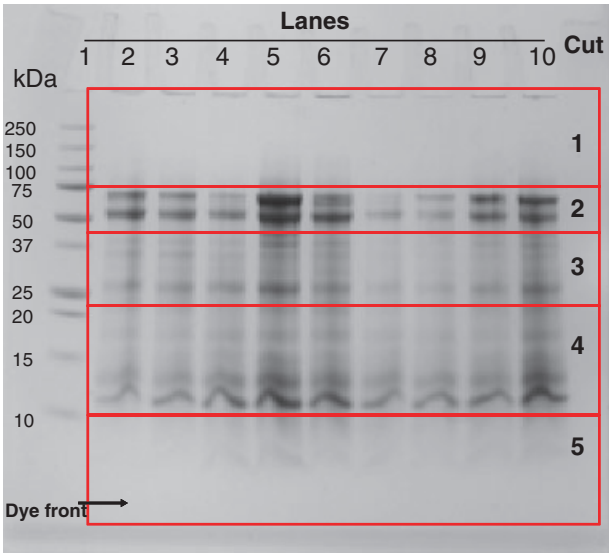

Fig. 3. SDS-PAGE eletro-elution of the gingival crevicular fluid proteins from periopaper strips. A picture of an SDS-PAGE, Coomassie blue-stained gel, showing an example of sectioning of the gel according to different molecular weight regions. All gingival crevicular fluid samples were from healthy sites. Lane 1, standard molecular weight proteins; lanes 2–10, nine individual periopapers run separately. On the right-hand side, under 'Cut', are the sections of different molecular weight regions excised across all five lanes and processed for MS analysis. Cut 1: relative molecular mass range ~100 kDa and above; cut 2, relative molecular mass range ~40–80 kDa; cut 3, relative molecular mass range ~25–38 kDa; cut 4, relative molecular mass range ~11–24 kDa; and cut 5, relative molecular mass range ~2–10 kDa.

NH_4HCO_3 and direct MS analysis after trypsin digestion, with no further separation step or sample simplification. This yielded identification of 84 proteins, of which 33 were distinct proteins not found by the above two approaches. The data obtained from all three different sample processing methods and MS analyses are summarized in Table 1, in which the proteins are arranged and categorized according to their biological groups.

In total, these multidimensional approaches led to identification of 199 distinct proteins in gingival crevicular fluid from periodontally healthy individuals. Of interest is the fact that only 14 proteins could be found common to all three methods used in this study (Fig. 4), whereas each of the approaches could give additional new and distinct proteins not found in another and with some overlapping proteins.

We have also established during these studies that the volume of gingival crevicular fluid from periodontally healthy patients was 0.49 ± 0.12 μL per site per periopaper strip and had a protein concentration of 96.38 ± 36.51 mgr;g/μL total protein, as determined by a modified micro-Lowry's protein assay (46), consistent with previous reports (35). The serum albumin concentration was ~15 μg/μL in gingival crevicular fluid samples measured by ELISA and, assuming that albumin represents ~50% (w/w) of the total serum proteins, an estimate of other serum proteins in gingival

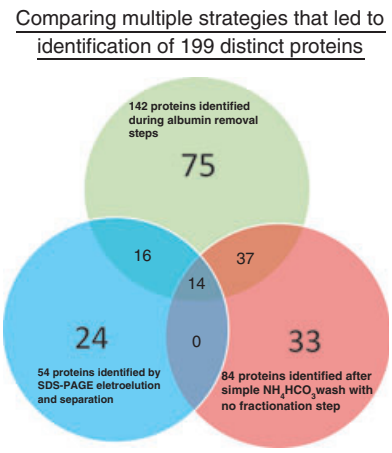

Fig. 4. Venn diagram summarizing the total of 199 proteins identified in the gingival crevicular fluid of healthy sites by multidimensional mass spectrometry. The diagram shows the distinct sets of proteins found only in each of the different experimental approaches and analyses (nonoverlapped regions) and those that are common (overlapped regions).

crevicular fluid is ~15 μg/μL of gingival crevicular fluid, implying ~30 μg/μL (w/w) total serum protein gingival crevicular fluid of periodontally healthy sites. Hence, of the total protein composition in gingival crevicular fluid from healthy sites, only a small portion (~30 μg/μL; ~30%) was serum derived, and the remaining 66 μg/μL was specific to the local gingival crevicular fluid microenvironment. Clearly, the availability of only a very small sample volume is compensated by the relatively high concentrations of proteins in this biological fluid. Other investigators have also successfully collected gingival crevicular fluid from clinically healthy sites for studies using classical biochemical methods, since the 1970s and 1980s (36,37). Apart from the expected serum-derived proteins, the data revealed the presence of sets of macromolecules more specific to the local gingival crevicular fluid microenvironment and not identified in the plasma proteome by MS technology. These proteins included those specific to early inflammation, the immune response and the innate immune response, specific bactericidal proteins involved in the defense response to bacteria, proteins that regulate production of cytokines such as interleukin-6 and interleukin-8 and regulate macrophage activity and tumor necrosis factor production, cellular and extracellular matrix components such as collagens, a variety of enzymes including proteinases (cathepsin G, leuckocyte elastase, leukitriene A4 and amidase) and an array of proteinase inhibitors.

The presence of serum-derived proteins in gingival crevicular fluid even from healthy sites generates a major interest in comparing and defining what proportion and which specific proteins found in gingival crevicular fluid by this large-scale proteomic approach could be serum related and which are specific to the gingival crevicular fluid microenvironment. This has twofold significance, on the one hand related to the establishment of biomarkers for systemic diseases and on the other to reveal local gingival crevicular fluid microenvironment-specific proteins with potential for the development of periodontal disease biomarkers. This comparison was possible because the plasma proteome has been published using MS analytical methods (22), permitting us to establish a comparative correlation with our gingival crevicular fluid proteome. This analysis resulted in 105 proteins (57%) of our gingival crevicular fluid proteome that were also identified in plasma by MS and, surprisingly, about half (98 proteins; 43%) were apparently not plasma related. Hence, we refer to these latter set of proteins as the gingival crevicular fluid microenvironment-specific proteins. These results are summarized in Table 1, with distinguishing labels for each protein and its possible origin, i.e. found in serum (S) or found only in gingival crevicular fluid. Table S1 provides the sequences of the peptides identified for each protein, their charged states, cross-correlation scores, and the molecular function and biological process of each protein. These results reinforce the idea that even though serum contributes to the composition gingival crevicular fluid, the gingival crevicular fluid from healthy periodontium sites is neither pure serum nor are its proteins all serum derived. It should be noted that the identification of a given protein in both gingival crevicular fluid and plasma does not exclude its potential as a diagnostic biomarker for locally based periodontal disease. A good example is provided by S100A8 and S100A9 proteins, which can be found in plasma, but their levels in gingival crevicular fluid may differ significantly during periodontal disease owing to the local inflammatory response and recruitment of neutrophils and macrophages, which are the cells responsible for their production. Interestingly, within the context of the identified proteins in gingival crevicular fluid of healthy periodontal sites, further evaluation of the data revealed important occurrences. Remarkably, the distribution of functional protein categories in the gingival crevicular fluid highlights a shift from almost equal distribution (~11% of each of the categories) for proteins that may be derived from serum to those distinct major categories for proteins specific to the gingival crevicular fluid microenvironment (Fig. 5). In particular, the dominant two categories of proteins specific to gingival crevicular fluid microenvironment were the intracellular and nucleotide proteins (25%) and the hydrolytic enzymes (19%; Fig. 5C). These observations are of significance because these two functional categories are directly related to periodontal disease and are highly likely to become significantly accentuated during disease progression. Likewise, in terms of cellular localization, the distribution of the identified proteins shows that the serum proteins were predominantly of extracellular origin (47%), with the next dominant being cytoplasmic (33%; Fig. 6B). However, the proteins specific to the gingival crevicular fluid microenvironment showed a different distribution, with the majority being cytoplasmic (39%) and next two major distributions being of extracellular (24%) and nuclear origin (22%). Note that the latter was only 9% for serum proteins (Fig. 6B,C).

The idea of using gingival crevicular fluid as a source of diagnostic biomarkers for periodontal disease is not new. For several decades, many potential periodontal disease biomarkers

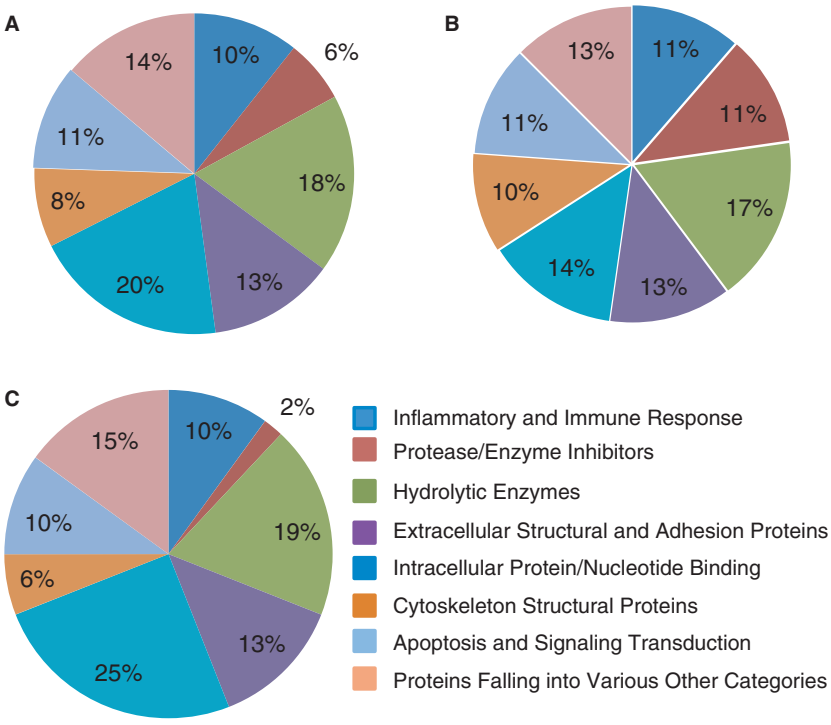

Fig. 5. Pie charts of the molecular and functional categories of the gingival crevicular fluid proteome from healthy periodontal sites. (A) Functional categories of the total 199 proteins identified within gingival crevicular fluid proteome. (B) Functional categories of the 105 gingival crevicular fluid proteins that can also be found in the plasma proteome. (C) Functional categories of the 94 gingival crevicular fluid proteins specific to the gingival crevicular fluid microenvironment.

have been screened, but all of these have been based on targeting a single analyte at a given time (10). In more recent times, there has been a paradigm shift towards the possibility of using a panel of independent disease-related proteins that may serve better and more accurately reflect the disease correlation. In this respect, the ability to highlight simultaneously a large number of proteins with local tissue/cell specificity becomes of major interest, which the present work fulfils. It is important to note that no classical and well-known glandular secretory proteins were identified in the present work in gingival crevicular fluid, indicating lack of contamination of the gingival crevicular fluid by saliva in the oral cavity during collection. Contrary to our work, however, Ngo *et al.* (13), using capillary glass collection method, found only a handful of distinct proteins plus many keratins by an MS approach in gingival crevicular fluid of periodontal disease pockets within which there were salivary contaminant proteins, such as statherin, acidic proline-rich proteins and histatins. Another recent study also used periopapers for collection of gingival crevicular fluid from periodontally inflamed sites and investigated relative quantitative changes in gingival crevicular fluid proteome levels as a function of time of experimentally induced inflammation (40). That study used a 21 d experimental human gingivitis model, in which inflammation was induced as a function of time by restricting brushing on selected sites. The study documented identification of 186 proteins and, when cross-correlated with the proteins in gingival crevicular fluid of healthy periodontium sites reported in the present study (Table 1), only 28 proteins were found to be common.

Conclusions

The results reported on the gingival crevicular fluid proteome using multidimensional protein separation and MS approaches enabled identification of a large number of proteins in the gingival crevicular fluid of healthy periodontium sites. Comparison of the studies using MS technology and gingival crevicular fluid from periodontally inflamed or periodontal sites (12,13,40) with our work highlighted important facets of such studies related to gingival crevicular fluid in that, with the exception of the most abundant proteins, very few overlaps existed amongst the proteins identified so far by MS technologies; hence, the present work clearly adds substantially to our knowledge of the gingival crevicular fluid proteome of healthy periodontal sites. This is likely to be due to differences in samples, healthy sites vs. inflamed and periodontal disease sites, the degree of fractionation of the protein samples, removal of abundant proteins such as albumin, and use of chemical labeling for quantitative work or direct qualitative proteome analysis. Furthermore, the present work also revealed that when gingival crevicular fluid is collected by periopaper, a simple elution of the proteins by NH_4HCO_3 leaves a significant number

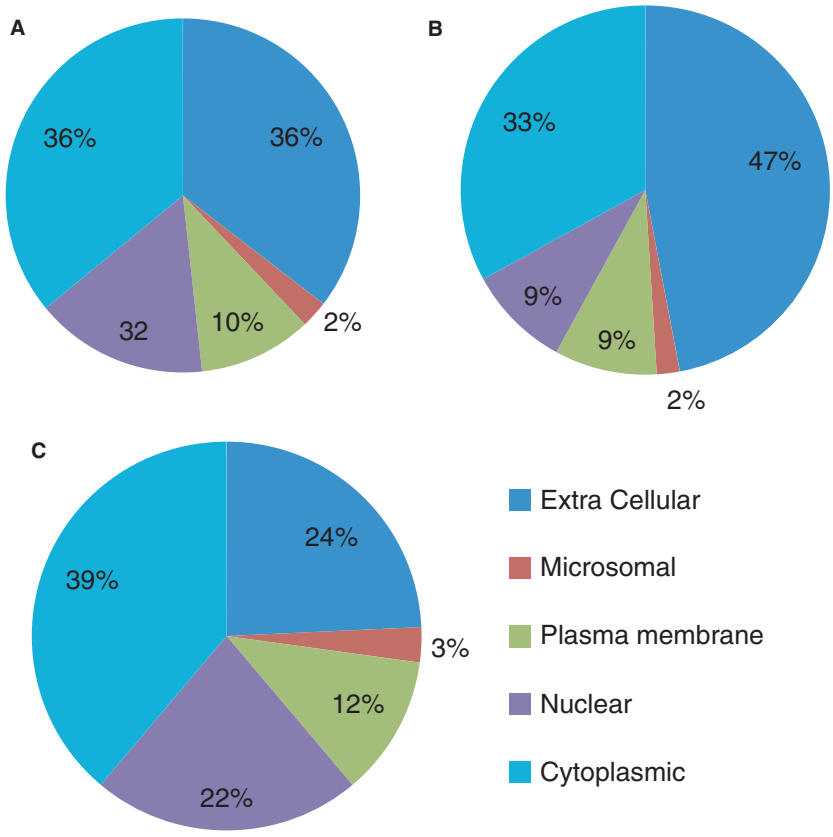

Fig. 6. Pie charts of distribution and cellular locality of the identified gingival crevicular fluid proteins. (A) Cellular distribution of the total 199 proteins identified within gingival crevicular fluid proteome. (B) Cellular distribution of the 105 gingival crevicular fluid proteins that could be also identified in the plasma proteome. (C) Cellular distribution of 94 gingival crevicular fluid proteins specific to the gingival crevicular fluid microenvironment.

of proteins on the periopaper, with some proteins not being eluted at all and others only partly. Therefore, caution should be exercised when such collection methods are used for relative quantitative proteomic studies. In comparison with other proteomic studies of saliva, such as parotid secretion in which more than 1100 proteins have been identified (28) and in whole saliva 450 (26) and 2300 proteins (39), the proteome of gingival crevicular fluid still remains in its infancy. A combined total of about 400 distinct proteins have been identified (~50% of which are serum derived) from the present work (199 proteins) plus that of Grant *et al.* (186 proteins, with 28 proteins common; 40), Bostanci *et al.* (101 proteins, with 56 proteins common; 11) and Ngo *et al.* (66 proteins, with 56 proteins common; 13). The cross-correlation of our data for gingival crevicular fluid and the plasma proteomes in the literature permitted dissociation of our 199 identified gingival crevicular fluid proteins into 105 proteins that can also be identified in plasma and 94 proteins that are likely to be specific to the gingival crevicular fluid microenvironment. In this respect, the importance of the gingival crevicular fluid proteome from periodontally healthy sites can be appreciated, because gingival crevicular fluid is the major link between 'serum' (systemwise fluid) and 'oral cavity' (whole saliva). In essence, our data provide another dimension and augment ongoing gingival crevicular fluid proteome studies towards forming the basis for the development of diagnostic biomarkers for health vs. disease states.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Gingival crevicular fluid proteome, 199 proteins, documented for healthy periodontium sites. The proteins have been grouped into seven different categories based on their known biological function(s).

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