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## Collection, Storage and Protein Extraction Method of Gingival Crevicular Fluid for Proteomic Analysis

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### Abstract:

Gingival crevicular fluid (GCF) may reflect the events associated with orthodontic tooth movement. Attempts have been conducted to identify biomarkers reflecting optimum orthodontic force, unwanted sequelae (i.e. root resorption) and accelerated tooth movement. The aim of the present study is to find out a standardized GCF collection, storage and total protein extraction method from apparently healthy gingival sites with orthodontics that is compatible with further high-throughput proteomics. Eighteen patients who required extractions of both maxillary first premolars were recruited in this study. These teeth were randomly assigned to either heavy (225g) or light force (25g), and their site specific GCF was collected at baseline and after 1hr, 1day, 7days, 14days, 21days and 28days post force application. Periostrips were used for GCF collection and subsequent phosphate buffered saline (PBS) was used for immediate protein elution with centrifugal speed of 10000rpm for 5min and stored at -80°C. Protein concentration was estimated using Bradford colorimetric assay. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to resolve the purity of proteins in the collected samples and the method of collection was validated by western immuno-blotting of alpha amylase salivary enzyme. The current collection, storage and protein extraction protocol showed the best protein recovery and purity with validated collection free of salivary contamination. In conclusion, tiny GCF volume from healthy sites and evaporation issues of such promising non-invasive fluid motivate us to investigate a standardized protocol enabling optimal preservation of GCF sample and the currently followed protocol may serve as a reference for future proteomic studies searching for GCF biomarkers in diagnosing and monitoring orthodontic tooth movement.

**Key words:** Biomarker, Gingival crevicular fluid, Orthodontic force, Proteomic, SDS-PAGE.

### Introduction:

The potential use of GCF markers proves to be a non-invasive method employed to clinically monitor and evenly enhance the efficiency and effectiveness of orthodontic treatment thus avoiding adverse sequelae associated with orthodontic treatment, such as root resorption or bone loss<sup>1</sup>. The high incidence and severity of unwanted consequences to optimum continuous applied orthodontic force<sup>2</sup> claims for the need of incorporation of basic research validated by scientific evidence with routine clinical practice to minimize such unwanted and iatrogenic biological consequences of orthodontic treatment itself. Over the last two decades, many investigations have been done on GCF biomarkers during orthodontic tooth movement to identify disease activity and risk<sup>3</sup>.

Improved knowledge of humoral immune responses, genomics, proteomics and more recently metabolomics has broadened the prospective applications of GCF analysis. Applications of proteomic tools revolutionized various biomedical disciplines such as genetics, molecular biology, medicine and dentistry. Proteomics is instrumental in discovery of biomarkers, that indicates a particular physiological, pathological and/or pharmacologic treatment condition<sup>4</sup>. Oral fluids including unstimulated whole mouth saliva (UMWMS) and GCF have been studied more for proteomic analysis due to their non-invasive collection technique, minimal patient discomfort and anxiety as compared to blood collection for serum or plasma. Compared to other bio-fluids, GCF has been analysed extensively for protein

profiles to explore periodontal health status as a fluid lying in close proximity to periodontal tissue, GCF is the principal target in researches for proteomic biomarkers discovery of periodontal diseases. However, a rising number of studies has recently used the expression proteomics to identify proteins whose abundance levels are altered by a disease using two-dimensional electrophoresis (2-DE) and followed by the state-of-art mass spectrometry (MS) to monitor changes that occur in the protein component of biological samples<sup>5</sup>.

Pre-analytical and analytical factors, concerning GCF collection and processing, could significantly influence quality and reproducibility of site-specific markers prediction and interpretations<sup>6,7</sup>. The standardization of pre-analytical and analytical procedures of GCF analysis is a challenging task. The minute volume of GCF harvested in healthy sites is a serious drawback affecting the results of analysis, as well as the specific equipment and tools required for GCF sampling which are considered as another limitation.

Nevertheless, the proteomic analysis is quite a challenging task due to the complexity of biological structures and physiological processes, rendering the path of exploration paved with various difficulties and pitfalls. Moreover, proteomics has not yet been fulfilled for clinical useful biomarkers discovery, because of the challenging analytical problems on the discovery side, and, on the validation side, the high cost and difficulties of developing specific immunoassays<sup>8</sup>. However, current proteomic tools allow large-scale and high throughput analyses of data, thus allowing to resolve thousands of proteins simultaneously and lead to many new discoveries but still to be the tip of iceberg<sup>9</sup>.

Currently, molecular testing kits with standardized procedure of sample collection and processing have not yet been produced to diagnose the orthodontic sequel on a clinical basis. To achieve this goal, there is still a need for more high-resolution protein research methods to look for new molecular markers. The technique of choice for this task is one-dimensional SDS-PAGE, as a protein-extract quality evaluation step followed by two-dimensional electrophoresis (2DE) and mass spectrometry<sup>10</sup>. Thus, this study attempts to optimize the method of GCF collection from healthy gingival sites, storage and total protein extraction method that is suitable for the high-throughput 2DE gel-based proteomic analytical techniques.

## Material and Methods:

### Participants and Orthodontic Procedure

Eighteen patients (7 men and 11 women) aged between 18 and 32 years' old, who required extractions of both maxillary first premolars as part of orthodontic treatment, were recruited in this study. They did not have any systemic disease, gingivitis, periodontal disease or tooth decay and extraction. In addition, they did not take any systemic medication for at least the last three months from their enrolment. Detailed written informed consent was obtained from each participant before the study commenced. Ethics approval was obtained from Human Research Committee of College of Dentistry/ University of Baghdad [with the protocol ID: 023418, No: 23 on January 9, 2019] and have a retrospective registration ID: NCT04291443 in ClinicalTrials.gov/ U.S. National Library of Medicine on March 2, 2020.

Self-ligating brackets with a 0.022" × 0.028" slot size and MBT system (Orthometric®, Brazil) were bonded with Transbond (3M Unitek, USA) on both maxillary 1<sup>st</sup> premolars after tooth surface etching using 37% Orthophosphoric acid-etch gel (Condac 37, FGM Dental Group, Brazil). Sectional orthodontic wires with different force magnitudes were randomly assigned to either right or left maxillary 1<sup>st</sup> premolar. Thus, one premolar received experimental buccally directed heavy force (225g) generated from 0.019" × 0.025" of beta-titanium molybdenum alloy (TMA) wire (Ormco Inc, Orange CA, USA), and a light force (25g) made from 0.016" TMA was applied on the other side to serve as control. There was no reactivation to the spring along the study period. All clinical procedures were carried out at the postgraduate orthodontic clinic of college of dentistry/ university of Baghdad from November 2018 and finished in June 2019, with 7 follow-up points for each participant which are baseline, 1hr, 1day, 7days, 14days, 21days and 28days before extraction.

### GCF Collection

GCF samples were collected from participants at seven time points during the day between 9:00am and 2:30pm using Periostrip (OraFlow Inc. New York, USA) (Fig. 1A), from four sides of each premolar (mesiobuccally, distobuccally, mesio palataly and distopalataly). Firstly, plaque was removed gently by cotton, then teeth were gently dried by air syringe in occlusal direction and the surrounding area isolated with cotton-wool rolls. The following steps were followed:

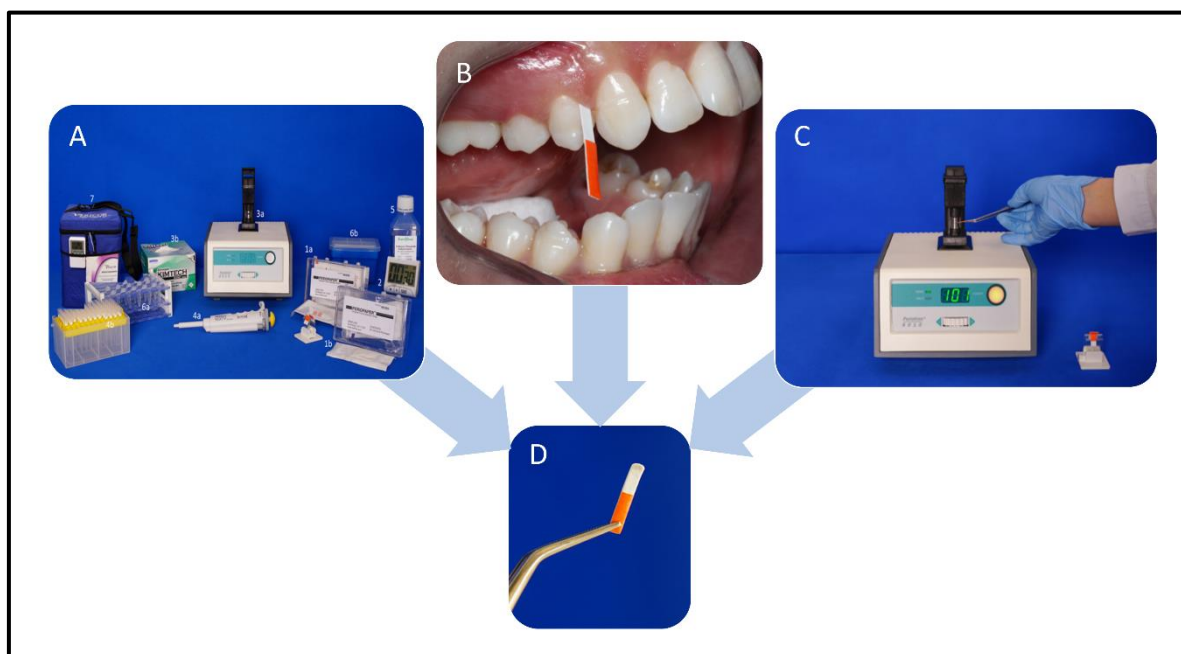
1. Periostrip placed 1mm sub-gingivally into the healthy gingival crevice for 30sec with 1min

interval (Fig. 1B). If there was any contamination of the strip with saliva or blood, it was discarded.

2. The volume of the collected GCF was immediately scored using Periotron 8010 (OraFlow Inc. New York, USA) electronic micro-moisture meter (Fig. 1C), by placing the strip between the two upper and lower electrode, then closing the lever brings the two electrodes together and begins a measurement cycle. After a pre-set time, the Periotron displayed a number, which was referred to

as a Periotron Score which needs to be transferred using software program (Periotron Professional 3.0) to represent the amount of fluid on the paper strip.

3. Each strip was placed in an individual Eppendorf Tubes® (Promega, USA), of 500µl capacity while the handle of the strip was secured under the lid and kept for further processing and preparations for protein extraction (Fig. 1D). The experiment workflow is described in Fig. 2.



**Figure 1. Steps for GCF collection for proteomic analysis. A, the equipment and devices needed for GCF collection, handling and transporting to be stored until time of analysis. B, Periostrip placed 1mm sub-gingivally into the healthy gingival crevice for 30sec. C, Periotron 8010 needed to estimate the volume of the collected GCF immediately. D, collected GCF on each periotrips ready for processing and fluid elution.**

### Protein Extraction and Storage

Protein contents in each strip were eluted with 20µL of Dulbecco's PBS (Euroclone®, S.P.A., Italy), then immediately transferred to a cooling box (Cool Cube™ 03, VericorMedLLC, Holmen, USA) at 2 to 4°C. The collected samples were carried to the laboratory where they were processed by centrifugation at 10000rpm for 5min. Then the eluted GCF of 4 strips per each tooth was aspirated and pooled into a new Eppendorf tube resulting in 80µL/tooth and 18 GCF samples of 80µL per each side, thus a total of 252 GCF samples were stored at - 80°C in freezer until the time for proteomic analysis. This process was carried out according to a previous study conducted by Saloom (2017) <sup>11</sup>.

### Bradford Assay for Protein Quantification

To determine the respective protein content of GCF from each side, the eighteen eluted samples were collected to form a pool of proteins from each

group, a Bradford protein assay was performed (Bio-Rad Laboratories, Richmond, CA, USA). A standard curve has been developed by 7 Bovine Serum Albumins standard dilutions (Biorad Laboratories, Richmond, CA, USA), from 0-2 µg/µL. In a 96-well plate, 5µL of GCF sample and standard solution were applied in each well, followed by 250µL of Bradford reagent added and incubated for 5min at room temperature. Every well's absorption was measured at 595nm using the multi-mode Biotek Synergy™ 2 microplate reader (Biotek Instrument Inc., Winooski, USA). A simple linear regression model with a squared r was used to develop the standard curves using the equation  $y=ax+b$  [a is the slope and b represents the y-axis crossing points]. By this equation, the concentration of protein (µg/µL) correspondent to each absorbance reading obtained from each sample

dilution was estimated. Protein concentration was determined in triplicate.

### One-Dimensional Polyacrylamide Gel Electrophoresis

To evaluate the quality of protein content of collected GCF samples, one-dimensional SDS-PAGE was applied as described by Laemmli<sup>12</sup>. Prior to PAGE, 2 $\mu$ g of each sample was mixed with SDS reducing buffer (0.5M Tris-HCl, pH 6.8, 10% SDS, Glycerol, and Bromophenol Blue) at a ratio of 1:1 and denatured at 95°C for 5min. Samples were then electrophoresed in a 5% stacking gel followed by 12% resolving gel at 100V for 50min, using Mini-Protean III System (Biorad Laboratories, Richmond, CA, USA). After SDS-PAGE is completed, the gels were stained using silver staining method as described by Yan *et al.* (2000)<sup>13</sup>. Each gel contains a marker in the first lane, Precision Plus Protein™ Dual Color Standards (Biorad Laboratories, Richmond, CA, USA) was loaded to estimate the molecular weight of resolved proteins. Gels were stained with silver nitrate staining solution, on a shaker, at room temperature, for 6hr. Later on, the gels were de-stained with de-staining solution (40% methanol, 7% acetic acid in H<sub>2</sub>O) for 40min.

### Validation of GCF Collection Using Western Blotting

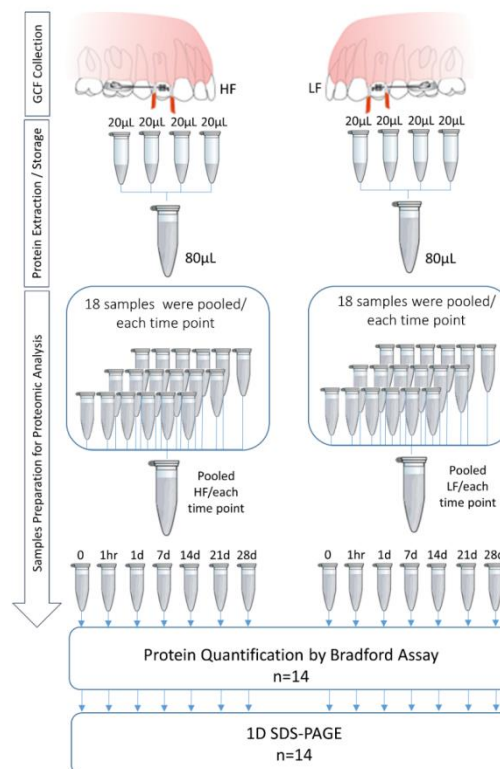
The proper collection of GCF has been optimized by detection of certain biomarkers including amylase; saliva alpha amylase enzyme is entirely originated from salivary gland and around 80 percent of it is secreted by the parotid glands and the remainder by submandibular glands<sup>14</sup>. Therefore, in the current study, it was used as a control to check the validity of GCF sample collection without saliva contamination. A total of 10 $\mu$ L of unstimulated whole mouth saliva (UWMS) and eluted GCF, with an equal amount of total protein (2.25 $\mu$ g) was applied to SDS-PAGE gel. Proteins were transferred onto a nitrocellulose membrane by Western blot and immuno-blotting.

For Western blotting, an X-cell vertical electrophoresis unit was used for packing the SDSPAGE gel with a nitrocellulose membrane which was sandwiched between filter paper and the sponge that has been saturated with 25mL of NuPage transfer buffer (Invitrogen, ThermoFisher Scientific, USA), 50mL methanol and 425mL deionized water. Then, the unit was assembled, secured and filled with the remaining transfer buffer. Transfer parameters used were 30V constant and 150mA for 60min. Once the transfer was finished, the gel was extracted and the membrane positioned in a sterilized tray for protein detection by immunoblotting.

For immunoblotting, Tris Buffered Saline with Tween was used as a blocking solution to block the membrane with agitation on a shaker for 1hr. A rabbit polyclonal 2B (pancreatic) IgG antibody [N<sub>3</sub>C<sub>3</sub>] (Gene Tex Inc, Alton Pkwy Irvine, CA, USA) was used as primary antibody at a dilution of 1:1000 which was added to the membrane gently. The membrane was washed with blocking solution three times, 5min each. Following washing, the membrane was incubated with a secondary antibody diluted at 1:2000 with blocking solution for 1hr with gentle rocking. The washing process was repeated as described previously. Chemiluminescent immunoassay (Immuno-Star Western C, Biorad Laboratories, Hercules, CA, USA) was used for detection of antibody signal and chemi Doc™ Imaging system (Biorad Laboratories, Hercules, CA, USA) was used to visualize the results.

### Statistical Analysis

Demographical statistic including (mean, standard deviation SD and standard error SE) was used to describe the results of protein concentration of the collected GCF in different force groups at different time-points (0day, 1hr, 1day, 7days, 14days, 21days and 28days).



**Figure 2.** The study workflow showing the preparation of collected GCF from each side with elution and pooling for proteomic analysis.

**Results:**

**Protein Concentration Quantification by Colorimetric Bradford Assay**

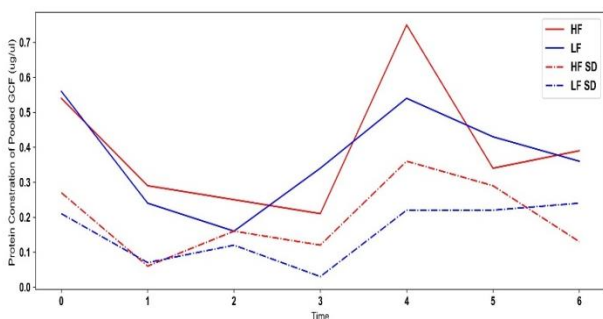
For each pooled samples at each time-points, a Bradford assay was carried out to estimate their protein concentrations, which are essential for the determination of the amount of proteins needed for rehydration of immobiline pH gradient strips before isoelectric focusing

experiment in 2DE electrophoretic proteins separation. The results of protein concentration showed gradual decrease in protein level after force application in both sides. This is followed by sharp increase after 14days reaching its maximum level ( $0.75\pm 0.21\mu\text{g}/\mu\text{L}$ ) in heavy force side. Furthermore, protein concentration was reduced after 28days at the end of the trial in both sides, (Table 1, Fig.3).

**Table 1. Protein concentration ( $\mu\text{g}/\mu\text{L}$ ) of pooled GCF samples estimated by Bradford assay of both force side at seven time-points.**

Group/Time	N	Protein Concentrations $\mu\text{g}/\mu\text{L}$			
		Minimum	Maximum	Mean $\pm$ SE	SD
HF0	3	0.35	0.85	0.54 $\pm$ 0.16	0.27
HF1	3	0.24	0.36	0.29 $\pm$ 0.04	0.06
HF2	3	0.07	0.37	0.25 $\pm$ 0.09	0.16
HF3	3	0.07	0.31	0.21 $\pm$ 0.07	0.12
HF4	3	0.34	1.00	0.75 $\pm$ 0.21	0.36
HF5	3	0.10	0.66	0.34 $\pm$ 0.17	0.29
HF6	3	0.29	0.54	0.39 $\pm$ 0.08	0.13
LF0	3	0.39	0.80	0.56 $\pm$ 0.12	0.21
LF1	3	0.19	0.32	0.24 $\pm$ 0.04	0.07
LF2	3	0.06	0.29	0.16 $\pm$ 0.07	0.12
LF3	3	0.31	0.37	0.34 $\pm$ 0.02	0.03
LF4	3	0.37	0.79	0.54 $\pm$ 0.13	0.22
LF5	3	0.26	0.68	0.43 $\pm$ 0.13	0.22
LF6	3	0.12	0.60	0.36 $\pm$ 0.14	0.24

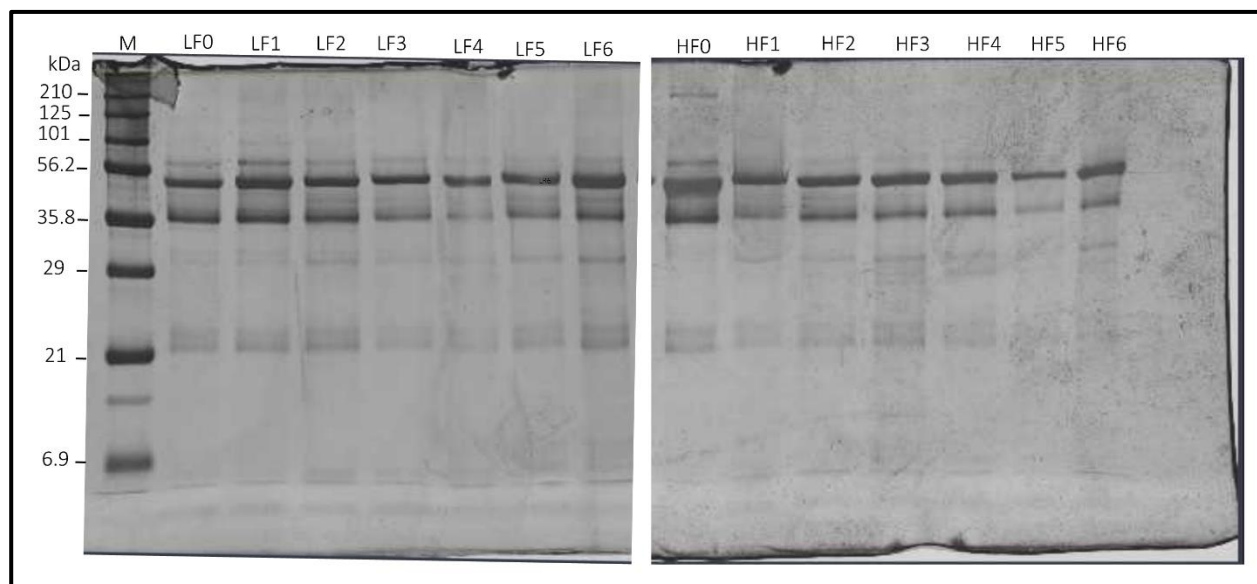
HF, heavy force; LF, light force; 7 time-points. [0, baseline; 1, 1hr; 2, 1day; 3, 7days; 4, 14days; 5, 21days and 6, 28days]. SE, standard error of mean.



**Figure 3. Simple linear graph showed protein content in pooled GCF samples of both force group at seven time-points.**

**GCF Proteins Purity by SDS-PAGE**

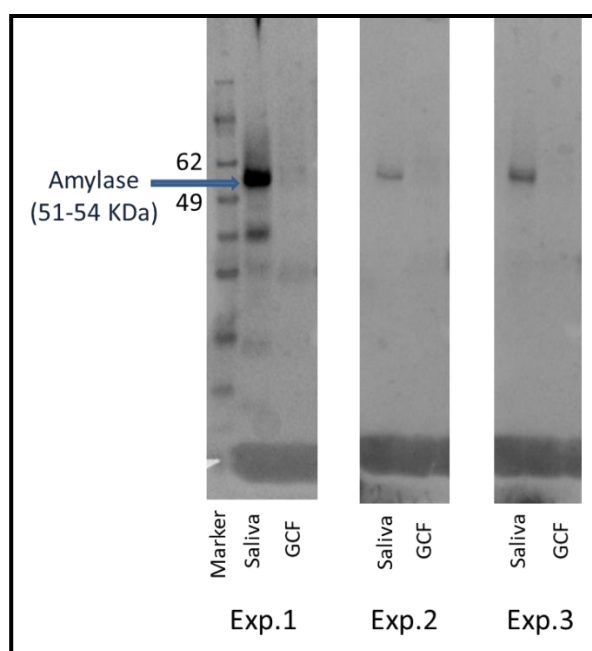
The resolution of protein bands was satisfactory in terms of sharpness and amount of bands. Proteins purity of pooled GCF samples were assessed by SDS-PAGE for different force groups (heavy and light-force), as shown in Fig. 4. The intensity of resolved band patterns on gels gave initial information on the quantity and quality of protein contents in the collected samples. Protein bands profiles of each pooled samples were similar, that is they scattered between 21 and 101kDa with different intensity. The more-intense bands were particularly resolved on 35.8 and 56.2 area, compared to the others.



**Figure 4.** SDS-PAGE profiles of pooled GCF samples showed bands of proteins purity of collected samples at different time-points. HF, heavy force; LF, light force; 7 time-points. [0, baseline; 1, 1hr; 2, 1day; 3, 7days; 4, 14days; 5, 21days and 6, 28days]. M, marker with range of molecular weight 6.9-210kDa.

#### Western Blot and Immunoblotting of Collected GCF

Saliva alpha amylase enzyme was used for evaluating the method of proper GCF collection by paper strips. The results from a total of three experiments at a dilution of 1:1000, the collected samples of GCF Western blotting showed that amylase was present in UWMS and absent in GCF, (see Fig. 5), which confirms that there were no saliva contaminants in the collected GCF samples.



**Figure 5.** The three experiments for immunodetection of alpha amylase (at molecular weight 51-54kDa) in UWMS and GCF.

#### Discussion:

This study was conducted to determine valid and standardized procedure for the collection, storage and protein extraction method of GCF for further gel-based proteomic analysis. GCF is a better diagnostic choice for assessing bio-molecules and mediators of health and disease, assuming that to its simple and non-invasive collection in comparison to blood as well as its sensitivity, convenience and repetitiveness<sup>15,16</sup>. In the last decades, proteomic experiments in GCF, saliva and gingival tissues have been carried out to discover a proteomic signature of healthy and diseased periodontal tissue<sup>6,7</sup>. In orthodontics, clinicians claimed to get an ideal result with optimum force magnitude and minimum adverse effect with lower time of treatment. Many researchers have therefore studied the profile and the levels of various enzymes, cytokines, growth factors and proteoglycans, primarily in the oral fluids (GCF and saliva), in order to monitor orthodontic tooth movement with its subsequent reactions in humans without any invasive impact<sup>2,17</sup>.

According to Preianò *et al.*<sup>6,7</sup>, GCF protein signature may be affected by major issues including standardization of the sampling, preparation and maintenance of the stability of proteome for this oral fluid. Therefore; in the present study, we proposed standardized steps of GCF collection validation, protein extraction and concentration estimation for protein purification of GCF content by SDS-PAGE, in order to be followed to distinguish a characteristic protein signature and

profiling using further high-throughput proteomic technologies.

In consistency with the results of the previous collection protocols and processing methodology, the major concern in GCF experiments is the collection of GCF from healthy subjects because of its minute volume and vaporization issues. To date, the influence of different sampling procedures and the effects of pre-analytical and analytical conditions on GCF proteomic profiling have not been thoroughly investigated in the proteomic studies which analyzed this biological fluid. The major issue facing GCF collection from healthy gingiva is the extremely low GCF flow rate<sup>18</sup>, meaning that the collected GCF volume is critical in a validated sampling period. Furthermore, a long sampling period can cause discomfort and bleeding due to the existence of shallow sites<sup>6</sup>, so collection time in the present study was set at 30sec to decrease the likelihood of blood contamination. In addition, due to the relatively small volume obtained in such a limited period from a single healthy site, it was appropriate to collect a pool of four sites per tooth.

Despite their nil effect on GCF proteome as suggested in the previous study<sup>6</sup>, the circadian rhythms for its collection were established at the same time in the morning (9:00am-2:30pm) and by the same experienced operator in order to diminish possible factors affecting results. Different methods were available for GCF collection such as capillary tubing, crevice washing and absorbent filter paper. Depending on its collection from healthy rather than inflamed gingival sulcus, the easier, convenient and non-traumatic method is the use of filter paper<sup>19</sup>. Furthermore, paper strips with a U-shaped end are more preferable than paper points for their larger contact area allowing more fluid absorption and reducing the risk of bleeding<sup>6</sup>.

In GCF proteomic studies, one of the important issues in GCF collection is the potential salivary contamination because of high proteases content<sup>20</sup>. Therefore, many previous studies have validated methods of GCF collection without salivary contamination. Griffith *et al.* (1992)<sup>21</sup> suggested a good method for isolating and drying, that has been followed in the current study, where the likelihood for salivary contamination can be neglected. In a recent study<sup>11</sup>, the researchers have optimized its accurate collection by western blotting of salivary alpha amylase; which accounts for about 20% in weight of salivary proteins and it is the most abundant protein in whole saliva<sup>20</sup>. The results showed that there was no amylase present in GCF, thus confirms validating its collection method.

However, in gel-based GCF proteomic studies, the process of sample extracting from paper strips is the most critical step in achieving reliable, accurate and reproducible results. Standardized sample preparation should be able, without any structural or chemical alteration, to solubilize and extract proteins, to remove interfering compounds and to keep the sample consistent with further proteomic analytical process<sup>22</sup>. GCF research has also recorded several other conflicting findings because of the used eluents, the pH, paper strip materials, the different protein sample concentrations and the procedural adjustments (elution measures, centrifuge speed and time). In reviewing the available literature details, the most suitable method for sample preparation is immediate elution with isotonic neutral PBS (pH=7.4) followed by centrifugal procedure, as this eluent fluid provides a satisfactory and full protein recovery from the strips without the addition of any other detergents from lysis of the strips materials<sup>23,24</sup>.

In a previous study, GCF was eluted from Periopaper with 20 $\mu$ L PBS with centrifugal time 5min at 9200g speed<sup>11</sup>, which was considered to be the optimal elution condition for GCF collected by paper strips because neither the Periopapers destroyed at 16300g of three tested times (5, 10 and 15min), nor the remained wet may have been fully eluted following centrifugation at 5 and 10min with 5900g and at all three tested times with 2300g centrifuging. Therefore, in the current study, centrifuging was adopted at 10000rpm for 5min as intact and dry Periopapers were obtained without any broken down. On the other hand, the elution volume is also another conflicting factor as the more diluted samples will impair protein concentration in the sample thus flawed results will be obtained. Thus, the elution volume has been adopted from previous studies to be set at 20 $\mu$ L per each strip resulting in a total of 80 $\mu$ L per each tooth and approximately 1440 $\mu$ L of GCF samples pooled from 18teeth per side at each time point. The results of protein concentration estimation by Bradford colorimetric method indicated that the volume of elution is validated by reflecting the immediate effect of force application on permeability of periodontium that affected protein concentration reaching its peak after 14days of force application related to peaked proteins ration of bone remodeling process showed to be initial bone resorption followed by a late phase of bone deposition (7-14days) marked by an increase in bone deposition proteins.

Another critical issue in GCF proteomic analysis is the storage condition -20 vs -80°C, in the

current study, the collected samples after centrifuge were stored at  $-80^{\circ}\text{C}$  as recommended by a previous study<sup>7</sup>. Comparing protein profiling of GCF and the results showed that more intact peptidome and proteome of recovered and resolved samples are better to be stored at  $-80^{\circ}\text{C}$  rather than  $-20^{\circ}\text{C}$ .

Different protein analysing methods are available to recover and resolve the highest number of proteins from the sample, SDS-PAGE gel electrophoresis is a widely used, simple and inexpensive method for resolving proteins in complex mixtures, first described by Laemmli (1970)<sup>12</sup>. SDS-PAGE has proven its biochemical effectiveness in the maintenance of protein structure without any protein denaturation<sup>25</sup>. Therefore, for very small and critical sample as GCF, a preliminary one dimensional protein profiling is recommended proceeding high-throughput, more sophisticated and expensive proteomic analysis to evaluate protein purity of the collected samples. The results of current SDS-PAGE revealed different bands of proteins with different molecular weight in the collected samples at different time points indicating the purity of the samples and its availability for further proteomic analysis such as ELISA or mass spectrometric analysis.

The tiny volume of GCF sample collected from healthy sites and evaporation issues of such promising non-invasive fluid motivate us to investigate a standardized protocol enabling optimal preservation of GCF sample and the currently followed protocol may serve as a reference for future proteomic studies searching for GCF biomarkers in diagnostic and monitoring of orthodontic tooth movement.

## Conclusion:

In conclusion, the current results highlighted the results of previous studies on the issues of the conditions associated with the collection, elution, storage and recovering of protein content in the collected GCF for further proteomic analysis. We conclude the following:

1. Paper strips ended with u-shaped can be used as proper collection method of GCF from healthy sites.
2. 30sec with 1min interval for subsequent collection is the best sampling time to collect enough sample size of such tiny sample.
3. 20 $\mu\text{L}$  of PBS could be the more preservative elution fluid resulting in full protein recovery from the strips of GCF sample.
4. Immediate centrifugation with 10000rpm for 5min results in intact and dry Periopapers without any break down.

5.  $-80^{\circ}\text{C}$  GCF storage condition shows more intact proteome purity resolved by SDS-PAGE.
6. The colorimetric Bradford assay is quite sufficient to estimate protein concentration in GCF samples.

In view of these crucial points, a standardized method has been implemented to quickly obtain reproducible proteome GCF profiling from healthy sites of clinically healthy subjects.

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## Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

## Authors' contributions statement:

**Harraa S. Mohammed-Salih:** Conception, design, acquisition of data, analysis, interpretation, drafting and writing.

**Hayder F. Saloom:** Conception, design, interpretation, revision and proofreading.

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## طريقة جمع وتخزين واستخراج البروتينات من السائل اللثوي لاستخدامه في التحليلات البروتينية

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### الخلاصة:

قد يعكس السائل اللثوي (GCF) الأحداث المرتبطة بحركة الأسنان التقييمية. تم إجراء محاولات لتحديد المؤشرات الحيوية التي تعكس قوة تقويم الأسنان المثلى ، والعواقب غير المرغوب فيها (مثل ارتشاف الجذر) وطرق واساليب تسريع حركة الأسنان . ان الهدف من هذه الدراسة هو انشاء طريقة مثلى لتجميع وتخزين واستخلاص البروتين الكلي القياسي من السائل اللثوي من مواقع اللثة الصحية ظاهريا من مرضى تقويم الأسنان تتماشى مع الطرق المتطورة لتحديد وتشخيص البروتينات كمؤشرات حيوية. تضمنت الدراسة ثمانية عشر مريضاً اللذين يحتاجون إلى قلع كل من الضواحك الأولى للفك العلوي والتي تم تعيينها عشوائياً لتسليط القوة إما للقوة الثقيلة (225 جم) أو القوة الخفيفة (25 جم) ، وتم جمع السائل اللثوي لفترات زمنية متعددة بعد ساعة واحدة ويوم واحد و 7 أيام و 14 يوماً و 21 يوماً و 28 يوماً من بدء التجربة. تم استخدام اشرطة لثوية لجمع السائل، اما محلول الفوسفات الملحي (PBS) لاستخلاص وانتزاع البروتينات من هذه الشرائط بسرعة طرد مركزي تبلغ 10000 دورة في الدقيقة لمدة 5 دقائق وتخزينها عند -80 درجة مئوية. أجري اختبار برادفورد لتقدير تركيز البروتين ، ثم تم إجراء التحليل الكهربائي لهلام دوديسيل كبريتات الصوديوم (SDS-PAGE) لمعرفة درجة نقاء البروتينات في العينات التي تم جمعها وتم التحقق من صحة طريقة الجمع عن طريق النشاف المناعي الغربي لإنزيم اللعاب ألفا أميليز. أظهر بروتوكول الجمع والتخزين واستخلاص البروتين الحالي أفضل النتائج لاستخلاص البروتين ونقاوته مع مجموعة مثبتة خالية من التلوث باللعاب. وتوصلت الدراسة الى ان حجم السائل الصغير من المواقع الصحية ومشاكل التبخر لمثل هذا السائل الواعد يحفزنا على التحقيق في بروتوكول جديد يتيح الحفظ الأمثل لعينة السائل اللثوي وقد يعمل البروتوكول المتبع حالياً كمرجع للدراسات البروتينية المستقبلية التي تبحث عن المؤشرات الحيوية لسائل اللثوي في تشخيص ومراقبة حركة الأسنان التقييمية.

**الكلمات المفتاحية:** المؤشر الحيوي ، السائل اللثوي ، قوة تقويم الأسنان ، البروتينوم ، دوديسيل كبريتات الصوديوم.