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Original article

Proteomic analysis of gingival crevicular fluid for novel biomarkers of pubertal growth peak

Xi Wen[1,2], Lorenzo Franchi[3], Feng Chen[2,4,*] and Yan Gu[1,2,*]

[1]Department of orthodontics, Peking University School and Hospital of Stomatology, No. 22 Zhongguancun Avenue South, Haidian District, PR. China, [2]National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology, No. 22 Zhongguancun Avenue South, Haidian District, PR. China, [3]Department of Orthodontics, University of Florence, Italy, [4]Central Laboratory, Peking University School and Hospital of Stomatology, No. 22 Zhongguancun Avenue South, Haidian District, PR. China

*Both authors have made an equal contribution.

Correspondence to: Yan Gu, Department of orthodontics, Peking University School and Hospital of Stomatology, No. 22 Zhongguancun Avenue South, Haidian District, Beijing 100081, PR. China. E-mail: guyan96@126.com; Feng Chen, Central Laboratory, Peking University School and Hospital of Stomatology, No. 22 Zhongguancun Avenue South, Haidian District, Beijing 100081, PR. China. E-mail: moleculecf@gmail.com

Summary

Background: Detection of pubertal growth peak is vital in orthodontic treatment timing and planning. Gingival crevicular fluid (GCF) contains abundant proteins from different sources and is an ideal source of biomarkers. The aim of this research is to detect candidate GCF biomarkers of pubertal growth by tandem mass tags (TMT) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to help diagnosis and treatment planning of functional treatment.
Methods: Forty subjects were recruited and were divided into pubertal and post-pubertal groups according to cervical vertebral maturation method. GCF samples were collected by paper points. GCF proteome of pubertal and post-pubertal subjects was compared by TMT labelling coupled with LC-MS/MS.
Results: A total of 537 proteins were detected in GCF samples, with 183 proteins detected in GCF for the first time. These proteins were involved in processes of immune response, ion transport, and signal transduction. The GCF concentration of vitamin D binding protein (DBP) and seroserotransferrin (Tf) was significantly higher in pubertal than that in post-pubertal subjects. DBP and Tf, therefore, were considered to be candidate biomarkers of pubertal growth. This result was validated using GCF samples from new subjects ($P < 0.05$).
Conclusion: Our results indicate that TMT labelling coupled with LC-MS/MS were proved to be a useful method for proteomic analysis of GCF with high accuracy. The expression of DBP and Tf was increased in children at circumpubertal stage and can be considered candidate biomarkers of pubertal growth.

Introduction

Identification of skeletal maturation stage, especially the onset of pubertal growth, plays a vital role in decision of treatment timing of orthodontic treatment. Previous studies revealed that for Class II subjects with retrusive mandible, functional treatment by removable appliances would obtain a remarkable skeletal effect if the orthopedic treatment is performed at the pubertal growth peak (1,2). For Class III subjects, the effect of maxillary protraction would be more effective if it is initiated before the onset of the pubertal peak (3).

Traditional methods to identify pubertal stage are hand–wrist method (HVM) and cervical vertebral maturation (CVM) method, which could cause extra X-ray radiation and have high demand for

clinical experience (4,5). Researchers have made great efforts to establish a simple and non-invasive method to precisely detect the pubertal growth peak. New possibilities are provided by biomarkers in body fluid. A longitudinal study revealed that the amount of change in serum IGF-1 had positive correlation with the amount of mandibular growth in pubertal stage (6). However, the procedure of serum collection was invasive and could cause patients discomfort.

According to the theory proposed by Alfano and Pashley, gingival crevicular fluid (GCF) flowing between root cervix and healthy free gingiva was initially produced as a transudate, sharing similar protein concentration with interstitial fluid. However, under inflamed condition, GCF would change to become exudate which had a protein range more similar to serum (7). Since its capability of reflecting host reaction to periodontal inflammation and its non-invasive characteristics, GCF has been proved to be an ideal source of biomarkers indicating periodontal diseases (8), orthodontic tooth movement (9), and pubertal growth spurt (10). Perinetti *et al*. found that the activity of alkaline phosphatase (ALP) in GCF reached a twofold peak in pubertal phases than in non-pubertal phases, suggesting that ALP could be an indicator of pubertal growth spurt (10,11). Our previous work applied matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) to analyse peptidomic spectra of GCF and detected six peptides as candidate biomarkers of pubertal growth peak (12).

During the last few decades, due to the application of liquid chromatography-tandem mass spectrum (LC-MS/MS), proteomic analysis is revealed as an effective tool for biomarkers discovery. The combination of tandem mass tags (TMT) and LC-MS/MS plays a vital role in comparative proteomics (13). Although TMT was widely used in searching for biomarkers in body fluids such as serum (14) and saliva (15), few studies applied TMT for GCF biomarkers discovery (13), and the whole protein profile of GCF has not been thoroughly discovered.

Hence, in our study, we used TMT labelling combined with LC-MS/MS to identify the whole GCF proteome of healthy subjects and to detect candidate biomarkers of pubertal growth peak through comparing GCF protein profiles between pubertal and post-pubertal subjects, to help orthodontic treatment planning.

Materials and methods

Subjects

Forty patients seeking orthodontic treatment at Department of orthodontics, Peking University Hospital of Stomatology from June to August 2016 were recruited in our study. All adults and parents of peadiatric subjects gave their informed consent. All protocols have been approved by Biomedical Ethics Committee (2016-NSF-01, 24 February 2016) and conform to standards indicated by the Declaration of Helsinki.

The inclusion criteria were as follows: 1. children at circumpubertal stage and young adults, 2. periodontal health, 3. no history of orthodontic treatment, 4. good general health without systematic diseases, 5. had not taken any medication for 3 months before the start of the study, and 6. Mongolian.

All subjects took lateral cephalograms as routine pre-treatment examination to determine the pubertal stage. According to the CVM method proposed by Franchi (16), subjects at CS 3 or 4 were included in pubertal group (10 males and 10 females, mean age 9.2 ± 1.4 years). On the other hand, subjects at CS 5 or 6 were included in post-pubertal group (10 males and 10 females, mean age 23.3 ± 0.64 years). Inter-observer agreement on CVM stages of all subjects was tested by two different orthodontists (XW and YG). Intra-observer agreement was determined by the same orthodontist (XW) initially and 1 week later. The investigators were blinded to information and chronological age of all subjects during the test.

Prior to GCF collection, clinical examination of each group was conducted to determine their periodontal status. Those who had attachment loss were excluded from the study. All subjects accepted ultrasonic scaling and oral hygiene instruction 1 week before GCF collection. Clinical parameters including probing depth, clinical attachment loss, and bleeding index were recorded before GCF collection. Only those who had probing depth 3 mm or less, no attachment loss and bleeding index 1 or less were included as studied subjects.

GCF collection and sample preparation

GCF was collected between 8–10 am. GCF samples were collected from mediolabial and distolabial sites of upper central incisors by paper points (DaYaDing, Tianjin, China). Sample sites were dried carefully and were isolated from saliva contamination by cotton rolls. Paper points were gently inserted into the gingival sulcus until minimum resistance was felt and left in place for 30 seconds. Paper points visibly contaminated by blood were discarded. The tips of four paper points were cut from where they were visibly wetted by GCF and were incubated in 30 μl 2.5 per cent TFA (HPLC grade, MACLIN, Shanghai, China) in the Eppendorf tube. After shaken for 10 minutes, the samples were centrifuged at 2000 × *g* for 10 minutes at 4°C. This procedure was repeated for three times and the elution solution was pooled together.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Twenty samples of each group were pooled together. Protein concentration of each group was determined by bicinchonininc acid (BCA) protein assay kit (CWBio, Beijing, China). An equal amount of proteins from each group (30 μg) was used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically separated on 12 per cent SDS-polyacrylamide gel at 100 mV for 2 hours. The lane between pubertal and post-pubertal lanes was chosen as control group. After electrophoresis, gels were stained with Coomassie brilliant blue and destained with 50 per cent methanol, 10 per cent acetic acid, and distilled water.

In-gel digestion and TMT labelling

Gel lanes of each group were cut into three pieces and were treated with 25 mmol/l dithiothreitol and 55 mmol/l iodoacetamide. In-gel digestion was applied using 12.5 μg/μl trypsin in 50 mmol/l ammonium bicarbonate at 37°C overnight. Peptides were extracted twice using 1 per cent trifluoracetic acid in 50 per cent acetonitrile aqueous for 30 minutes. TMT Isobaric Label Reagent Set (Thermo Scientific, USA) was used to label proteins and peptides according to manufacturer's protocols. Pubertal group, post-pubertal group, and control group were labelled with TMT-130, 127, and 126, respectively. Two microlitres TMT labelling reagent was added into each sample and incubated for 1 hour at room temperature, and 0.5 μl 5 per cent hydroxylamine was added to terminate reaction. Equal amounts of labelled proteins from each group were pooled together, dried, and solved in 0.1 per cent trifluoracetic acid for LC-MS/MS.

LC-MS/MS

TMT-labelled peptides were separated by Thermo-Dionex Ultimate 3000 HPLC system interfaced with Thermo Scientific Q Exactive

mass spectrometer (Thermo-Fisher Scientific, USA). Mobile phase A consisted of 0.1 per cent formic acid. Mobile phase B consisted of 100 per cent acetonitrile and 0.1 per cent formic acid. Mass spectrometry was performed in a data-dependent mode with single full-scan mass spectrum in the orbitrap (m/z 400–1800, 60 000 resolution), and MS/MS scans was carried out in data-dependent mode at 27 per cent collision energy. The raw data of MS/MS spectra were searched against the human Uniprot database using Proteome Discover (Thermo-Fisher Scientific, USA). The following search parameters were used: maximum of two missed trypsin cleavages, precursor ion mass tolerances set as 20 ppm, and fragment ion mass tolerance set as 20 mmu. At least two unique peptides per protein had to be identified to list the protein as a hit. The false discovery rate was set at 0.01. Protein ratios were calculated using the median of all peptide hits of one protein. A gene ontology (GO) analysis was carried out to Classify the whole GCF proteome and differentially expressed proteins between two groups.

Enzyme-linked immunesorbent assay of vitamin D binding protein and seroserotransferrin

Fifty microlitres phosphate buffered solution was added into the Eppendorf tubes containing four paper points and shaken at 4°C for 10 minutes, and then centrifuged at 9 000 rpm for 5 minutes. Total protein concentration of each sample was then determined by BCA protein assay kit (CWBio, Beijing, China). The concentration of vitamin D binding protein (DBP) and seroserotransferrin (Tf) in GCF was measured using commercially available ELISA kit (Qisong Company, Beijing, China) according to the protocols provided by manufacturer. The sensitivity of human Tf ELISA kit and DBP ELISA kit was less than 0.1 μg/ml and less than 1.0 ng/ml, respectively, with no significant cross-reaction with other soluble structural analogues. Three replicates were run for each sample and the mean value of their concentration was calculated for statistical analysis. The concentrations of DBP and Tf in GCF supernatant were normalized both per paper point and per μg of total protein.

Statistical analysis

All variables were normally distributed according to the results of Shapiro–Wilk test. Inter- and intra-observer agreement on CVM stages was tested by quadratic weighted kappa (AgreeStat 2015.6 for Windows). The differences of concentrations of DBP and Tf between pubertal and post-pubertal groups were analysed using independent samples *t*-test by SPSS software (version 19, IBM, USA). Differences at $P < 0.05$ were considered to be statistically significant.

Results

Forty subjects were included in our study, with 20 subjects in pubertal group and 20 subjects in post-pubertal group (Table 1). Lateral cephalograms of subjects in each group were shown in Figure 1A. Kappa values of inter- and intra-observer agreement for the CVM stages were 0.92 (95% confidence limits 0.86–0.99) and 0.97 (95% confidence limits 0.94–0.996), respectively. This result revealed almost perfect intra- and inter-observer agreement in the assessment of the CVM stages (17). GCF samples were collected by paper points in mesiolabial and distolabial sites of upper central incisors, as shown in Figure 1B. TMT labelling combined with LC-MS/MS was used for comparison of GCF proteome between pubertal and post-pubertal groups and the procedure was briefly illustrated in Figure 1C.

GCF proteome of healthy subjects

A total of 537 human proteins were detected in GCF samples. A filter criterion was set to screen out potential polluted proteins. Only proteins which satisfied all the following conditions were considered as GCF proteome: 1. Proteins which only existed in pubertal or post-pubertal group but not in control group, or proteins whose level in pubertal and post-pubertal groups were more than twice as control group; 2. scores 2 or more; and 3. peptide-spectral match (PSM) greater than 1. After screening out potential polluted proteins and proteins with low scores, 340 proteins remained and were considered as components of GCF proteome (Supplementary Table 1).

GO analysis was conducted to analyse the function of GCF proteome. As for biological process, innate immune response turned out to be the most important role of GCF played under healthy conditions (Figure 2A). We also analysed the molecular function (Figure 2B) and cellular component (Figure 2C) of GCF proteome, with a special concern with calcium ion binding proteins and extracellular exosome.

We compared the GCF proteome data identified in our study with four previous studies (18–21) and made a Venn diagram (Figure 2D). Among 340 proteins identified in our study, 157 proteins were overlapped while 183 proteins were detected in GCF for the first time, which was also the most among all studies.

Differentially expressed proteins between pubertal and post-pubertal groups

We selected differentially expressed proteins between pubertal and post-pubertal groups by setting criteria as follows: the proteins whose pubertal to post-pubertal ratios were more than or equal to 1.3 were considered as up-regulated proteins, whereas those proteins whose pubertal to post-pubertal ratios were smaller than or equal to 0.5 were considered as down-regulated proteins. Forty-three proteins were found to present different expressions between pubertal and post-pubertal groups, among which 25 proteins were higher and 18 proteins were lower in pubertal group (Table 2).

GO analysis of these differentially expressed proteins was conducted (Figure 3A). Among all Classifications, the term 'vitamin transport' was found to be the most significantly enriched ($P < 10^{-4}$). Interestingly, of all proteins related to vitamin transport, one was DBp. $1,25(OH)_2D$ is the active form of vitamin D and can influence mineral bone status (22) and promote intestinal calcium absorption (23). $1,25\text{-}(OH)_2D$ and its precursor 25-OHD were transported in

Table 1. Detailed information of studied subjects.

Group	Stage	Number	Chronological age (years)	Gender		Protein concentration of GCF (μg/ml)
				male	female	
Pubertal	CS3	13	8.8 ± 1.1	7	6	266.2 ± 75.6
	CS4	7	9.9 ± 1.6	3	4	220.1 ± 80.8
Post-pubertal	CS6	20	23.3 ± 0.6	10	10	295.6 ± 100.3

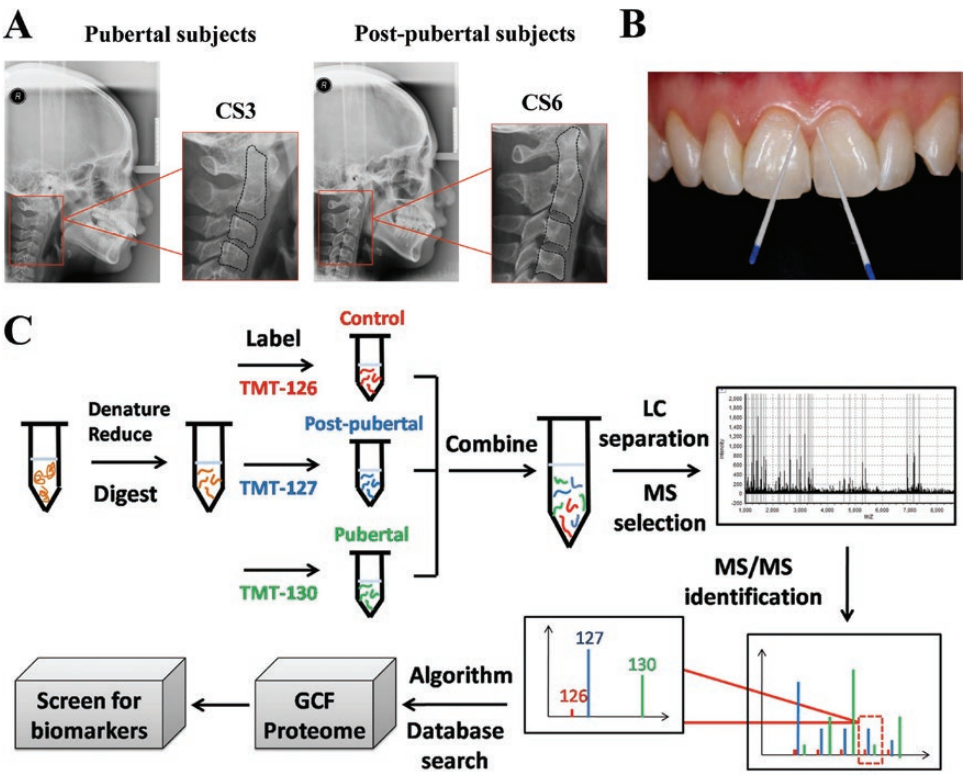

Figure 1. Brief illustration of dividing subjects, gingival crevicular fluid (GCF) collection, and mass spectrum analysis. (A) Lateral cephalograms of subjects in pubertal and post-pubertal groups. (B) GCF samples were collected by paper points in mesiolabial and distolabial sites of upper central incisors. (C) Brief illustration of TMT labelling combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS) to compare GCF proteome between pubertal and post-pubertal groups and screen for candidate biomarkers.

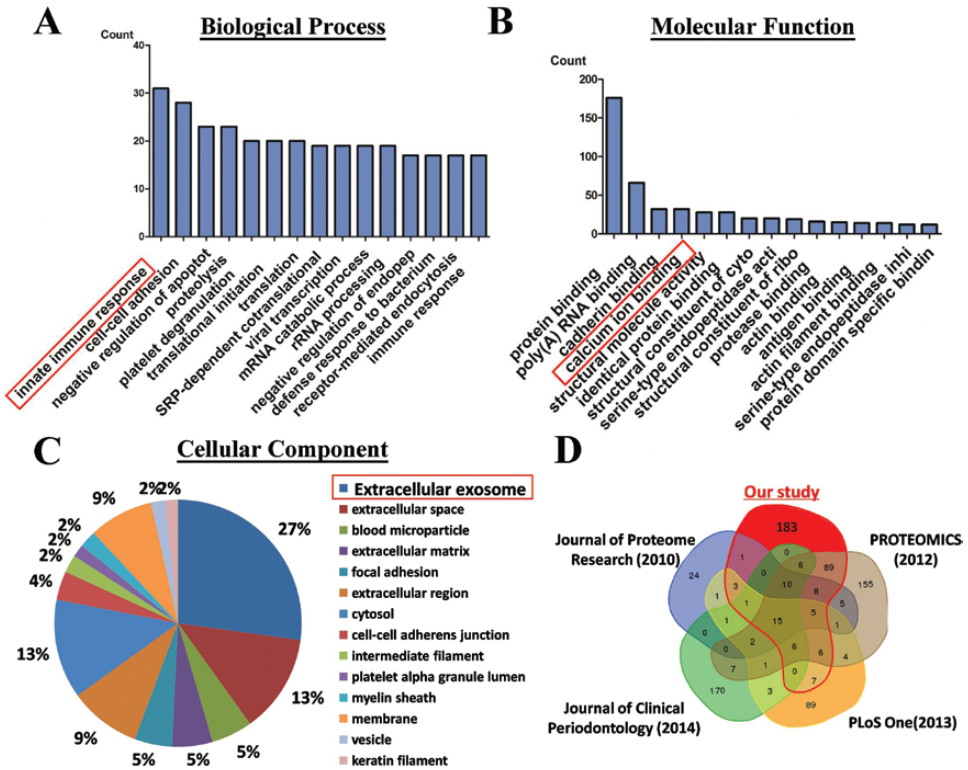

Figure 2. GCF proteome of healthy subjects. (A) Biological process of GCF proteome. Innate immune response turned out to be the most important role GCF played under healthy conditions. (B) Molecular function of GCF proteome. (C) Cellular component of GCF proteome. Most proteins in GCF (27 per cent) came from extracellular exosome. (D) Comparison of GCF proteome data between our study and four previous studies (Refs. 18, 19, 20, and 21). One hundred fifty-nine proteins were overlapped, whereas 183 proteins were detected in GCF for the first time, which was also the most among all studies.

Table 2. Differentially expressed proteins between pubertal and post-pubertal groups.

Accession	Description	Score	Coverage	# PSMs	Pubertal/post-pubertal	MW [kDa]
Proteins whose levels were higher in pubertal group						
P01023	Alpha-2-macroglobulin	56.13	14.31	25	1.790	163.2
P51884	Lumican	2.84	7.69	2	1.754	38.4
H7C2W9	60s ribosomal protein L31	4.45	28.70	3	1.696	12.8
P43652	Afamin	4.15	3.84	2	1.639	69.0
E9PFZ2	Ceruloplasmin	5.66	2.01	3	1.588	108.8
O60814	Histone H2B type 1-K	212.54	46.83	79	1.560	13.9
P02647	Apolipoprotein A-I	70.52	49.44	37	1.506	30.8
P01871	Ig mu chain C region	17.78	21.46	14	1.466	49.3
P04217	Alpha-1B-glycoprotein	13.57	11.31	5	1.462	54.2
P06899	Histone H2B type 1-J	195.17	46.83	74	1.449	13.9
P19652	Alpha-1-acid glycoprotein 2	31.22	21.89	14	1.438	23.6
P02768	Serum albumin	2148.71	72.25	756	1.396	69.3
P02790	Hemopexin	28.70	19.26	12	1.390	51.6
P01024	Complement C3	61.89	15.09	31	1.380	187.0
H7C5H1	Complement factor B (Fragment)	3.95	7.54	2	1.376	34.7
P02787	Serotransferrin	164.00	42.26	67	1.370	77.0
P02774	Vitamin D-binding protein	28.67	20.89	14	1.362	52.9
Q16777	Histone H2A type 2-C	76.24	55.04	30	1.357	14.0
J3QSB5	60S ribosomal protein L36	5.68	20.21	4	1.355	10.8
B7Z6M1	Plastin-3	5.82	5.81	3	1.350	65.6
Q96QV6	Histone H2A type 1-A	47.45	35.11	19	1.321	14.2
P39019	40S ribosomal protein S19	31.20	46.21	13	1.319	16.1
Q6P452	Annexin	2.93	9.03	2	1.308	33.5
P35579	Myosin-9	13.68	4.08	8	1.304	226.4
P31997	Cell adhesion molecule 8	17.00	9.74	9	1.304	38.1
Proteins whose levels were lower in pubertal group						
P61981	14-3-3 protein gamma	28.60	14.17	13	0.499	28.3
O00299	Chloride intracellular channel protein 1	4.15	11.20	2	0.493	26.9
H0Y5H9	Serpin B4 (Fragment)	25.92	22.10	20	0.484	42.5
P16403	Histone H1.2	370.10	41.78	141	0.479	21.4
P02538	Keratin, type II cytoskeletal 6A	1246.63	57.98	460	0.478	60.0
P62328	Thymosin beta-4	16.91	45.45	7	0.471	5.0
Q5SQ17	Chloride intracellular channel protein 3	5.29	15.57	2	0.469	18.8
P15259	Phosphoglycerate mutase 2	2.15	9.88	3	0.452	28.7
P02533	Keratin, type I cytoskeletal 14	1126.37	70.13	393	0.434	51.5
Q9HCY8	Protein S100-A14	16.63	50.96	9	0.421	11.7
F8W7S5	Ribosome-binding protein 1	4.25	1.73	2	0.389	84.3
P08779	Keratin, type I cytoskeletal 16	884.62	69.34	322	0.380	51.2
P31947-2	Isoform 2 of 14-3-3 protein sigma	51.71	49.54	29	0.376	24.3
P12035	Keratin, type II cytoskeletal 3	290.95	23.73	132	0.356	64.4
Q04695	Keratin, type I cytoskeletal 17	472.87	60.88	191	0.329	48.1
Q01546	Keratin, type II cytoskeletal 2 oral	365.69	40.91	158	0.296	65.8
B3EWG3	Protein FAM25A	4.98	25.84	3	0.289	9.3
P04259	Keratin, type II cytoskeletal 6B	1182.25	57.98	437	0.289	60.0

circulation bound primarily to DBP (24). Similarly, Tf in term 'iron ion homeostasis' transported iron ion in circulation between sites of absorption, storage, and utilization. Its function in transporting iron ion in circulation was similar to that of DBP in transporting vitamin D and was reported to be improved in serum of pubertal subjects (25). Therefore, we chose DBP and Tf as candidate biomarkers identifying pubertal growth peak.

Levels of DBP and Tf in GCF were significantly higher in pubertal than those in post-pubertal group

We collected GCF samples from another five pubertal subjects and five post-pubertal subjects to validate the results through ELISA. Total protein content of each GCF sample was 22.38 ± 1.23 μg. The concentrations of DBP and Tf in GCF supernatant were normalized both per paper point and per μg of total protein. Results are shown in Figure 3B. The levels of DBP and Tf in GCF were significantly higher in pubertal than those in post-pubertal group before and after normalization ($P < 0.05$).

Discussion

The issue of CVM reproducibility is controversial in the literature. The papers by Gabriel *et al.* (26) and Nestman *et al.* (4) report poor reproducibility. It should be noted that in both these studies, it was not clearly reported how the observers were trained in the CVM method. There is no doubt that in order to perform the CVM method correctly (as for any method or index to assess individual skeletal maturity like the HVM), the observer needs to be trained

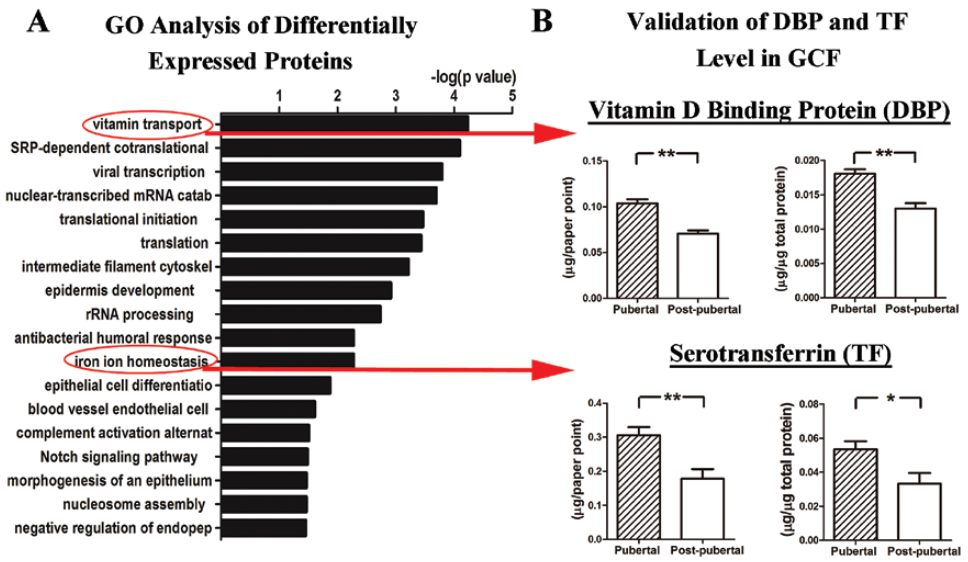

Figure 3. Levels of vitamin D binding protein (DBP) and seroserotransferrin (Tf) in GCF. (A) Classification of differentially expressed proteins between pubertal and post-pubertal groups by GO analysis. The term 'vitamin transport' was found to be the most significantly enriched ($P < 10^{-4}$). Another term related with molecular transportation is 'iron ion homeostasis'. DBP in 'vitamin transport' and Tf in 'iron ion homeostasis' were chosen as candidate biomarkers. (B) ELISA results showed that levels of DBP in GCF were significantly higher in pubertal than those in post-pubertal group, normalized by per paper point (pubertal: 0.103 ± 0.010 μg; post-pubertal: 0.071 ± 0.008 μg) and by total GCF protein (pubertal: 0.018 ± 0.001 μg; post-pubertal: 0.013 ± 0.002 μg; $N = 5$, $P < 0.01$). Levels of Tf in GCF were significantly higher in pubertal than those in post-pubertal group, normalized by per paper point (pubertal: 0.305 ± 0.054 μg; post-pubertal: 0.178 ± 0.063 μg) and by total GCF protein (pubertal: 0.053 ± 0.011 μg; post-pubertal: 0.033 ± 0.014 μg; $N = 5$, $P < 0.05$).

calibrated appropriately. This concept is very well illustrated in the paper by Perinetti *et al.* (27) who found that when specific training is provided along with precise guidelines in assessing visually each stage, the CVM method proves to be accurate and repeatable to a satisfactory level. In the present study, all observers were adequately trained in the CVM method by an expert examiner. The kappa value of both inter- and intra-observer agreement was almost perfect (0.92 and 0.97, respectively). The validity of the CVM method to detect the individual skeletal maturity is also a controversial topic in the literature. Soegiharto *et al.* (28) showed that the CVM method can be used as a valid clinical diagnostic index for the prediction of peak growth of the maxilla and the mandible. Similarly, a recent systematic review by Cericato *et al.* (29) reported that the CVM methods analysed in the study were reliable to replace the hand–wrist radiograph in predicting the pubertal growth spurt.

GCF collection can be performed using three different methods: filter paper strips, paper points, and capillary tubes. GCF collection with capillary tubes appears to be an ideal method as it can acquire undiluted fluid directly from gingival crevice. However, the insertion of tubes into gingival crevice can be traumatic, and the transfer of samples from capillary tubes to EP tubes is difficult. Therefore, the choice of capillary tube was excluded from our study. Paper strips and paper points are both common and easy to perform methods collecting GCF. Preianò *et al.* found that the use of paper points or paper strips did not affect the number of mass peaks of GCF MALDI-TOF profiling from healthy sites of healthy subjects (30). Chen *et al.* performed both *in vivo* and *ex vivo* experiments to compare the recovery rate of paper strips and paper points in collecting GCF samples. Results showed that the recovery rate of both methods exceeds 90 per cent and no significant difference was observed (31). However, paper strips used in previous studies were cut into rectangular shape with sharp edges, which could easily cause bleeding in healthy sites. Thus, we chose paper points to collect GCF samples in our study, which is both convenient and less harmful to gingival tissues.

GCF proteome of apparently healthy subjects plays a key role in detection of disease-related biomarkers as it can serve as a reference or baseline when analysing the change of GCF proteome under pathological conditions. Previous studies have conducted LC-MS/MS analysis of GCF from healthy subjects both in qualitative level (18,32) and quantitative level (21,33), most of which were 'label-free' methods. Due to the limited volume of GCF, quantitative analysis of GCF proteome using isobaric mass tags has not been well explored (20). TMT labelling prior to MS analysis enables multiple samples to be analysed in the same LC-MS/MS procedure. Its advantage of detecting proteins with low concentrations has been proved and has been widely used in identification of biomarkers in serum (14) and saliva (15), but rarely in GCF. Tsuchida *et al.* applied TMT labelling and detected 609 proteins in GCF from patients with periodontitis (13). However, GCF proteome of healthy subjects was not clearly clarified in this study.

Therefore, in our study, we used TMT labelling and LC-MS/MS to identify the whole proteome of GCF from periodontal healthy subjects. A total of 340 proteins were considered as GCF proteome of healthy subjects, with 183 proteins identified in GCF for the first time. We drew a Venn diagram (Figure 2D) to compare the GCF proteome identified in our study with four previous studies. The number of proteins identified in our study was the largest. This result proves that TMT labelling prior to LC-MS/MS is a powerful tool for the identification of GCF proteome (34) and has the ability to discover the proteins with less abundance, which were more difficult to be found using conventional label-free methods. One hundred eighty-three proteins were detected in GCF of healthy subjects for the first time, including S100A2, S100A16, and annexin A4, which belong to S100 family and annexin family, respectively. These protein families were believed to play a role in maintenance of periodontal health, but still need further investigation (35).

Novel functions of GCF proteome were revealed by GO analysis. Results showed that innate immune response is the most important

role GCF played in periodontal healthy subjects (Figure 2A), which is consistent with the conclusion that GCF serves as a barrier against biofilm bacteria of subgingival plaque under healthy conditions (36,37). In addition to cellular components like neutrophils, GCF also contains key molecular components of innate immune response to bacterial infection, such as defensins, complement components, and anti-microbial peptides (33). These proteins and peptides decreased in GCF of periodontitis patients and were considered as biomarkers of periodontal disease progression. Among all molecular functions of GCF proteome, we paid a special attention to annexins family in 'calcium ion binding' for its relationship with bone development and mineralization (Figure 2B). Annexins are constitutive proteins of matrix vesicles and are highly expressed in mineralizing growth plate cartilage (38). Annexin-mediated Ca^{2+} influx into growth plate chondrocytes is a positive regulator of mineralization (39), whereas in annexin-A1 null animals, anomalous development and delayed intramembranous ossification of the craniofacial bone were observed (40). These results indicate that annexins detected in GCF might play a similar role in development of craniofacial bone but still need further investigation. As for cellular components of GCF proteome, our results showed that most proteins in GCF (27 per cent) exist in exosomes (Figure 2C). Exosomes are small vesicles produced by cells to transmit molecules into intercellular space. It has been confirmed that saliva has large amounts of exosomes (41). Proteins in salivary exosomes can be used as biomarkers in diagnosis of lichen planus (42) and oral cancer (43). However, seldom research has focused on exosomes in GCF by now. Our results might set a direction for future research.

Previous studies related with GCF proteomics mainly focused on detecting biomarkers of periodontal diseases such as chronic periodontitis (21), aggressive periodontitis (19), and experimental gingivitis (44), but little is known about GCF biomarkers of pubertal growth. In order to selected candidate biomarkers of pubertal growth, we applied TMT labelling to identify differentially expressed proteins between pubertal and post-pubertal groups. GO analysis was employed to analyse the functions of these differentially expressed proteins (Figure 3A). Among all Classifications, we chose DBP and Tf in 'Vitamin transport' and 'iron ion homeostasis', respectively, as candidate biomarkers.

A sample pooling strategy was applied in our study before TMT labelling and LC-MS/MS. As we know that there is a great individual variability in protein content of the GCF sample. The main purpose of pooling all samples in the same group together is to reduce the impact of individual variability on protein expression level as much as possible. Meanwhile, TMT labelling quantification method allows for concurrent identification of up to 10 samples in a single experiment (34), which makes performing TMT labelling on each individual sample in this study unpractical. Therefore, we decided to pool 20 samples from the same group together for TMT labelling quantification to preliminarily investigate the level difference between two groups. However, the disadvantage of these samples pooling strategy is that statistical testing of significance cannot be performed. Thus, more strict validation tests are needed to verify the results provided by TMT labelling quantification.

We collected GCF samples from another 10 subjects and ELISA was employed to validate this hypothesis. Results showed that DBP and Tf levels in pubertal subjects were significantly higher than those in post-pubertal subjects (Figure 3B).

DBP, also known as group-specific components (Gc), transports 25(OH)D from liver to kidneys and other organs to be further synthesized to 1,25(OH)2D, the active form of vitamin D. It has been proved that 1,25(OH)2D plays a vital role in promoting intestinal calcium absorption and regulating maturation and mineralization of bone (21). About 80–90 per cent of 25(OH)D and 1,25(OH)2D in serum are bound to DBP. Previous studies have revealed that 25(OH)D levels were positively correlated with circulating DBP levels (45,46), suggesting that the 25(OH)D level would be elevated as the DBP level increased. During pubertal growth, 1,25(OH)2D level reached the peak in response to the high requirement of calcium in this critical phase of bone development (47). However, there is no direct evidence that reveals the correlation between DBP level and pubertal growth. Our research proved that DBP levels in GCF were significantly higher in pubertal subjects than those in post-pubertal subjects, possibly due to the high requirement for 25(OH)D to be converted into 1,25(OH)2D to improve the intestinal calcium absorption in puberty. Further studies are needed to investigate the mechanism underneath.

Tf is also a kind of serum protein that transports iron between sites of absorption, utilization, and storage. A previous study proved that during puberty, especially for pubertal boys, the serum Tf levels were increased compared with pre-pubertal subjects (25). A positive correlation was found between the increment of haemoglobin and Tf. Thus, during puberty, there is a physiological increase in Tf due to stimulated erythropoiesis (25). Our research confirmed that Tf levels in GCF of pubertal subjects were significantly higher than those in post-pubertal subjects, presenting a similar trend as serum, which can be a candidate biomarker identifying pubertal growth.

Periodontium is highly vascular, facilitating factors of serum and periodontal tissues infiltrating into gingival sulcus, which was totally bathed in GCF (37). Therefore, GCF is the key to understand the biological process of metabolism underneath the periodontal tissue. Our study tried to identify biomarkers in GCF that can indicate craniofacial pubertal peak, which has a close relationship with remodelling and turnover of local alveolar bone. Still, there are some limitations in our study. Firstly, the sample size is relatively small. Secondly, the dietary factors such as the intake of vitamin D and iron were not taken into account when recruiting the studied subjects. And the downstream mechanism of these GCF biomarkers regulating the bone metabolism and growth still remains unclear. In the future, we will conduct a longitudinal study to further validate our results.

Conclusion

TMT labelling coupled with LC-MS/MS was proved to be a useful method for proteomic analysis of GCF with high accuracy. The expression of DBP and Tf was increased in children at circumpubertal stage and can be considered to be candidate biomarkers of pubertal growth.

Supplementary material

Supplementary material is available at *European Journal of Orthodontics* online.

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Conflict of Interest statement

None to declare.

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