Research Article

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MALDI-TOF MS profiling in the discovery and identification of salivary proteomic patterns of temporomandibular joint disorders

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Abstract: This research aimed to identify differences in polypeptide/protein profiles of the unstimulated whole saliva between patients with temporomandibular joint disorders (TMDs) and healthy individuals. A fraction of the polypeptides/proteins (<30 kDa) was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The spectra were recovered from methanoic acid/acetonitrile salivary samples overlaid with an α -cyano-4-hydroxy-cinnamic acid matrix in positive linear mode at an interval of 1,500–20,000 m/z mass acquisition range. The data were analyzed for the selection of characteristic peaks by using the ClinProTools 3.0 software. Discriminative classification models were generated by using a quick classifier, supervised neural network, and genetic algorithms. From the 23 peaks exhibiting the highest discriminatory power, the ten top-scored peaks with the area under the receiver operating characteristic >0.8 were selected. A panel of salivary markers that predicted the patients with TMDs was selected (2728.0, 4530.2, 5174.2, 5193.3, 6303.4, 6886.7, 8141.7, 8948.7, 10663.2, 10823.7 and 11009.0 m/z). Although carried out on relatively small datasets, the classification algorithm used in this study allows the differentiation between salivary samples from

subjects with TMDs and healthy individuals and confirms the usefulness of a proteomic profiling approach in the monitoring of the disease.

Keywords: saliva, temporomandibular joint disorders, MALDI-TOF MS, proteomics

1 Introduction

Temporomandibular joint disorders (TMDs) represent a heterogeneous group of pathologies associated with morphological and functional orofacial deformities [1–3]. Common symptoms include dysfunctions of the temporomandibular joint (TMJ), inflammation, dislocation, developmental anomalies, and neoplasia accompanied by orofacial pain [4,5]. Subjective and clinical signs of TMD are manifested in surrounding muscles, ligaments, bones, synovial capsules, connective tissues, and teeth. It has been found that multiple degenerative changes associated with TMD involve a number of proteins. Studies [6–9] have shown that various cytokines including interleukin-1 β , IL-6, and tumor necrosis factor- α in the synovial fluid (SF) of TMD patients can promote processes leading to inflammation and degeneration of bone and cartilage of the TMJ. Moreover, it has been demonstrated that IL-1 β -induced matrix metalloproteinase (MMP) expression is a key factor in the initiation of cartilage destruction by Wnt-5A [10]. Further, activated protein C can act as a trigger of MMPs expression in the SF of patients with osteoarthritis, thus being a factor involved in cartilage breakdown [11]. The function of endocrine gland-derived vascular endothelial growth factor/prokineticin-1 and typical chemokine receptor 2 (D6) in the development of TMD has also been discussed [12].

In clinical research, the whole saliva provides several advantages over the SF, mainly due to its easy and noninvasive collection. Moreover, it has been shown that a multitude of proteins with similar functions might

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be found in common among different body fluids [13,14]. Saliva is a complex mixture of the secretions from the salivary glands, which also provides valuable information about proteins of mucosal exudates, proteins derived from the gingival crevicular fluid [15], and exogenous oral microflora [16,17] and shares proteins transported from blood plasma [18].

TMD may induce pain extending over the pathological anatomic structure. The option of obtaining samples for TMD biomarker research such as saliva is more attractive than the lumbar puncture of cerebrospinal fluid or biopsy from the trigeminal nerve [19].

Novel evidence was provided that levels of the proinflammatory peptides found in saliva showed a significant elevation in the saliva of TMD patients. Increased salivary IL-1 β levels regardless of a fibromyalgia diagnosis were observed [20]. There is therefore a persuasive rationale for exploring saliva as an alternative diagnostic and prognostic medium in the TMD research [16,21].

In recent years, studies on the human salivary proteome aiming to identify both potential clinically relevant biomarkers and drug targets have provided a unique opportunity for improving the prediction of the risk of developing several disorders [22–24]. In particular, the detection of TMD can benefit from a preventive examination and the prediction of the outcomes and effectiveness of clinical treatment. In such a way, understanding the pathophysiology of TMD can also be increased.

Currently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in combination with advanced bioinformatics tools has made available the development of a faster, more efficient, and cost-saving untargeted proteomic method that relies on the identification of characteristic MS fingerprints for profiling potential biomarkers [25].

The project solution focuses specifically on monitoring the expression of the low molecular weight components of the salivary proteome in the mass range of 1,500–20,000 Da, which represents a promising source of potential biomarkers directly related to the TMD etiology. Due to the above-mentioned reasons, mass spectrometric analysis of the prefractionated saliva was the method of choice for the present study.

The MALDI-TOF technique was used to establish a rapid screening protocol for the differentiation between individuals with TMD and subjects without clinical manifestations of TMD. The protein profile of the saliva helped to indicate the risk of developing the disease during a preventive examination, could be used to

evaluate the effectiveness of different treatments, and might provide additional insights into the molecular mechanism of TMD and reveal potential salivary biomarkers.

2 Materials and methods

2.1 Patients and sample preparation

All salivary samples were collected from the 1st Department of Stomatology, Faculty of Medicine, University of P. J. Šafárik between January 1st, 2018, and May 30th, 2019. The 17 control samples of the unstimulated whole saliva were obtained from healthy donors with an average age of 31.4 years (range: 19–61 years). Saliva samples of the TMD group were obtained from 18 patients with an average age of 35.2 years (range: 19–67 years). The saliva samples from the TMD patients and the controls were randomized, and the identity of the subjects was protected.

2.2 Samples and handling procedures

Approximately 5 mL of the unstimulated whole saliva was accumulated by expectoration into a sterile tube. Volunteers were instructed to not perform any oral hygiene, eating, or drinking for at least one hour before saliva collection. Saliva secretion, during the collection of the sample, was not stimulated mechanically or chemically. During the collection, the whole saliva was kept on ice. Immediately after collection, the saliva was cleaned up by centrifugation at $12,000 \times g$ for 30 min at 4°C. The supernatant was immediately processed or frozen and stored at –80°C until analysis.

2.3 Salivary sample processing for the MALDI-TOF MS profiling

Saliva samples were prepared according to the procedure adapted [26]. A 1 mL sample of clear saliva was processed with a centrifugation filter device (Amicon® Ultra 30K device, Merck KGaA, Darmstadt, Germany) at $12,000 \times g$ for 30 min at room temperature. For each 800 μ L of saliva filtrate, 3,600 μ L of precooled ethanol

was added and mixed. The sample was briefly vortexed and pelleted by centrifugation at $12,000 \times g$ for 15 min at room temperature followed by brief centrifugation of the pellet at $12,000 \times g$ for 2 min. After drying at room temperature, 50 μ L of 70% methanoic acid (Sigma-Aldrich, St. Louis, MO, USA; methanoic acid/H₂O, 70:30, v/v) was added and vortexed until complete dissolution of the pellet. After that, 50 μ L of acetonitrile (Sigma-Aldrich, St. Louis, MO, USA) was added to the sample, and again it was vortexed. Next, the sample was centrifuged at $12,000 \times g$ for 2 min at room temperature to remove any insoluble material. The supernatant of each sample was saved for spotting.

2.4 Matrix preparation

A ready-made Bruker Biotyper MALDI-TOF MS (Bruker Daltonics GmbH, Germany) protocol was adopted for the matrix preparation. A volume of 1 μ L of salivary protein sample was placed onto an MTP 384 polished steel target (Bruker Daltonics GmbH, Germany) and air-dried at room temperature before being overlaid with 1 μ L of α -cyano-4-hydroxycinnamic acid solution (saturated matrix solution in acetonitrile/water, 1:1, v/v prepared with 2.5% trifluoroacetic acid) (Sigma-Aldrich, St. Louis, MO, USA). Each sample was spotted twice onto the target.

2.5 MALDI-TOF MS

All samples were subjected to MALDI-TOF MS (MALDI TOF/TOF MS, UltrafleXtreme, Bruker Daltonics GmbH, Germany) analysis. Mass spectra were gained in positive linear ion mode at an interval of 1,500–20,000 m/z mass acquisition range. The parameters were set as follows: matrix suppression, m/z 0–1,000; laser shots, $2 \times 1,000$; and calibrated between samples with a protein standard (Bruker Daltonics GmbH, Germany).

2.6 Data analysis and model generation

The ClinProTools (version 3.0; Bruker Daltonics GmbH, Germany) was employed for data analysis. The software provides an analytic framework for the design of models for the automatic classification derived from differences in MALDI-TOF MS spectra from both patient and control

cohorts and recognition of the potential biomarkers that are indicative of a specific disease state.

The peak list calculation was performed within the range from 1,500 to 20,000 m/z . To define the optimal model allowing the discrimination of the saliva samples, at first, raw data were pretreated as follows: baseline subtraction – top hat baseline, 10% minimal baseline width; smoothing – Savitzky Golay smoothing filter, width set to 4 m/z , smoothing cycles, 20; recalibration – 1,000 ppm maximal peak shift, 10% match to calibrant peaks, exclusion of not recalibrated spectra; total average spectra calculation – resolution, 300; peak picking on total average peak list – signal-to-noise threshold, 4; peak calculation – intensities. The pretreatment procedures applied to samples were executed automatically using default values without user involvement. A total average spectrum calculated from the preliminarily processed spectra improved the signal-to-noise ratio for a peak picking procedure as well as for allowing taking into account the peaks that might be neglected on single spectra. The pretreated data were later used for visualization, statistical analysis, pattern determination, evaluation, and spectra classification as well as for identification of particular peaks to differentiate between the examined groups.

The peak intensity differences were evaluated based on their statistical significance, from either a Student's *t*-test (for normal variable distribution) or a Wilcoxon test (for nonnormal variable distribution) as the statistical methods to select discriminative peaks. The Anderson–Darling test was used for testing for a normal distribution of peak intensities. In all experiments, the cut-off of statistical significance was considered to be $p \leq 0.05$.

Furthermore, the discriminatory quality for each assumed biomarker of the salivary polypeptide/protein ions was evaluated by the area under the receiver operating characteristic (ROC) curve (AUC) that graphically represents the specificity versus the sensitivity of a putative differential peak.

For the classification of mass spectra from the model generation classes of control and diseased saliva samples, the following mathematical algorithms were employed: quick classifier (QC), genetic algorithm (GA), and supervised neural network (SNN). A total of 20 peaks in the averaged spectra were used in the model generation. A GA for the generation of a model for the analysis of mass spectra contained the following parameters: ≤ 50 generations, ≤ 20 peaks in the model, 0.2 mutation rate, 0.5 crossover rate, varying random seed, and 5 neighbors. SNN parameters were used for model analyses and the selection of polypeptide/protein peaks accommodated $\leq 2,000$ generations with the number of

prototypes set to 5. A univariate sorting algorithm of a QC used the automatic detection procedure to pick the optimal number of peaks to be incorporated into the model. For prediction models, cross-validation and recognition capability as criteria of the robustness of the generated models were calculated. For all models being generated, the cross-validation rate was set to random mode with 5% spectra to leave out.

3 Results

A total of 35 samples of the whole saliva of individuals, including 18 patients with TMD and 17 healthy controls, were recruited for the MALDI-TOF MS analysis. Polypeptide/protein mass fingerprints of each sample at a mass to charge ratio (m/z) from 1,500–20,000 were generated and loaded into ClinProTools and processed (Figure 1).

After filtering the noise and clustering, a total of 104 unique protein peak clusters were identified. In a univariate statistical analysis, the top-scored 24 peaks exhibited significant differences between the two sets of samples ranked by p values from the nonparametric Wilcoxon test. The ROC curves were used to analyze the diagnostic capacity of these 24 peaks further. AUC for the evaluation of the discrimination of the groups was calculated. The 10 top-scored peaks ($AUC > 0.8$) were selected (Table 1).

Cluster analysis by 2D peak distribution for the best separating peaks for healthy control subjects and TMD patients at 5174.2 and 10823.7 m/z , respectively, was plotted according to peak intensity (Figure 2). Distribution of the peaks at m/z 5174.2 and m/z 10823.7 manifests their discriminating capability between samples from TMD patients and healthy subjects.

The ROC curves for the best separating peaks for healthy control subjects and TMD patients at 5174.2 and 10823.7 m/z are further illustrated in Figure 3.

Three algorithms: QC, GA, and SNN were tested for the generation of recognition models for the classification of the TMD patients to discriminate them from the healthy control individuals. The highest recognition capability and cross-validation values were critical in the selection of models with the best performance power (Table 2).

The SNN model exhibited a recognition capability of 97.2%, which is higher than 91.3% for the GA. The testing of the QC algorithm resulted in the lowest recognition capability of 83.2%. Both the QC and the GA classification models presented almost identical cross-validation at 75.8% and 73.5%, respectively. The SNN algorithm presented the highest cross-validation of

Figure 1: The average mass spectra of the salivary samples from TMD patients (green line graph) and healthy control (red line graph). x-axis, mass-to-charge ratio (m/z); y-axis, relative intensity.

84.3%. Thus, the model created using the SNN algorithm showed the best performance parameters compared to QC and GA and was consequently selected to find the most relevant series of discriminatory peaks.

The SNN model automatically selected 11 mass peaks from a total of 104 identified unique protein peak clusters in the m/z 2728.0–11009.0 range (Figure 4). In the analysis of neural networks, the integration regions matching peaks included in the model are represented by blue bars, excluded ones are depicted by gray bars. The eleven differential peaks/regions incorporated into the model are represented by red bars. Changes in signal intensities between the TMD group (green) and control group (red) of all specific peaks/integration regions generated by the SNN model are depicted on small graphs placed around the perimeter.

The Anderson–Darling test was used to answer the question of whether the data passed the normality test.

Table 1: ClinProTools peak statistical analyses of the most discriminating polypeptide/protein ions determined in the saliva of TMD patients and the control group

Index	Mass	p_W -value	p_{AD} -value	Control (mean $\pm$ SD)	TMD (mean $\pm$ SD)	AUC
45	5174.2	0.00330	0.00897	0.96 $\pm$ 0.40	1.79 $\pm$ 0.70	0.866013
88	10823.7	0.00386	0.00324	0.28 $\pm$ 0.07	0.49 $\pm$ 0.20	0.852941
79	9057.9	0.00386	<0.000001	0.71 $\pm$ 0.88	2.29 $\pm$ 1.99	0.852941
97	12621.6	0.00442	<0.000001	0.27 $\pm$ 0.11	0.59 $\pm$ 0.44	0.843137
102	13568.5	0.00703	<0.000001	0.30 $\pm$ 0.21	0.55 $\pm$ 0.36	0.830065
74	7847.2	0.00988	<0.000001	0.87 $\pm$ 0.45	1.99 $\pm$ 1.90	0.816993
49	5535.3	0.01030	<0.000001	2.36 $\pm$ 1.16	6.50 $\pm$ 5.89	0.813275
75	8141.7	0.01230	<0.000001	0.35 $\pm$ 0.11	0.89 $\pm$ 0.68	0.807190
69	7453.3	0.01370	0.0000134	0.83 $\pm$ 0.33	1.59 $\pm$ 0.86	0.800654
89	11009.0	0.00330	<0.000001	0.65 $\pm$ 0.46	1.92 $\pm$ 1.92	0.800654

p_W , p -value obtained through a Wilcoxon test; p_{AD} , p -value obtained through an Anderson–Darling test; intensities of the control and TMD data sets (mean $\pm$ SD); AUC, area under the ROC curve.

Figure 2: ClinProTools 2D peak distribution of polypeptide/protein with m/z 5174.2 (x -axis) and 10823.7 (y -axis) between healthy control subjects and TMD patients (in red and green, respectively). The discriminating attributes of the first two peaks from the list of p values. Ellipses correspond to 95% confidence intervals.

All eleven peaks showed p values ≤ 0.05 , indicating that the data do not follow a normal distribution. Therefore, the Wilcoxon test was preferred over the t -test to assess peaks as statistically significant, indicating their discrimination power. Finally, the peaks/integration regions put into the classification models for discrimination of TMD status are shown in Table 3.

4 Discussion

The ideal method for identifying TMD should be quick and effective, which is crucial for adequate curative

Figure 3: Plots of ROC curves representing the relationship between sensitivity and specificity of m/z peaks 5174.2 and 10823.7. Areas under the ROC curves (AUC) are 0.866013 and 0.852941, respectively.

Table 2: Cross-validation and recognition capability of algorithms for the differentiation of the TMD from the control set in the whole saliva samples

Algorithm	Peaks for model generation	Generated peaks	Cross-validation (%)	Recognition capability (%)
QC	20	1	75.8	83.2
SNN	20	11	84.3	97.2
GA	20	10	73.5	91.3

Figure 4: Location of discriminating peak masses (m/z 2728.0, 4530.2, 5174.2, 5193.3, 6303.4, 6886.7, 8141.7, 8948.7, 10663.2, 10823.7, and 11009.0) determined by the SNN. Red and green denote the control and TMD groups, respectively.

Table 3: Combinations of peaks (m/z) included in the generated classification models

QC		SNN		GA	
Peak/integration region	m/z	Peak/integration region	m/z	Peak/integration region	m/z
45	5174.2	21	2728.0	45	5174.2
		36	4530.2	46	5193.3
		45	5174.2	60	6303.4
		46	5193.3	65	6828.5
		60	6303.4	69	7453.3
		66	6886.7	75	8141.7
		75	8141.7	87	10663.2
		78	8948.7	88	10823.7
		87	10663.2	95	12472.4
		88	10823.7	97	12621.6
		89	11009.0		

management of TMD and could overcome several shortcomings of conventional approaches [27]. Recent reports have shown that MALDI-TOF MS profiling is a non-invasive, simple, and low-cost tool to characterize distinctive salivary peptide biomarkers from patients with e.g., s-ECC [28,29] and identifying candidates with accelerating tooth movement [30] or chronic periodontitis, oral cancer, and oral lichen planus [31].

In the present work, we aimed to recognize patients with TMD for the first time employing an arrangement that depends upon the automated use of MALDI-TOF MS coupled with statistical analysis to generate a salivary protein fingerprint reflecting the disease status.

We showed preliminary evidence of the feasibility of applying the MALDI-TOF MS technique for comparative analysis of the salivary protein profiles of healthy individuals and TMD patients.

The ClinProTools software was used for all data interpretation steps, including the generation of spectra pattern recognition models, which helped us to prove the specificity of the salivary spectra of TMD individuals in comparison with those of healthy subjects. This fact was supported by a set of peaks that represent the MS patterns of both groups. A total of 104 unique protein peak clusters were significantly differentially expressed. The best predictive SNN model provided eleven peptide ion signatures (m/z 2728.0, 4530.2, 5174.2, 5193.3, 6303.4, 6886.7, 8141.7, 8948.7, 10663.2, 10823.7, and 11009.0) with a recognition capability of 97.2% and cross-validation of 84.3%. As a diagnostic panel, there are mainly two peaks of 5174.2 and 10823.7 m/z with an AUC value of 0.866013 and 0.852941, respectively, representing good accuracy in discriminating the diseased state. These findings point to the possibility of the application of this classification model as a sensitive and specific diagnostic tool and to reduce the complexity of classifiers, decreasing the possible interlaboratory variations and allowing for more feasible clinical strategies.

To our knowledge, no previous studies have employed MALDI-TOF MS-based proteomic techniques to research the association between saliva and TMD. None of the potential biomarker peaks have been previously reported or validated for the saliva of TMD patients.

The several drawbacks of this research must also be noted. Because of the relatively small size of the groups of subjects involved, follow-up studies are necessary to confirm the results and facilitate their evaluation and validation. Moreover, the study is limited by the absence of sequence identification of the peptides from the discriminating peaks that will specify the protein biomarker candidates involved in the MS pattern

changes caused by TMD, which needs to be conducted in future investigations.

Extending the MS saliva spectrum database, it can become a part of the Bruker MALDI Biotyper System (Bruker Daltonics GmbH, Germany) and used for the rapid classification of TMDs.

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Ethical approval: The study protocol was approved by the Ethics Committee of Louis Pasteur University Hospital in Košice (permission 2017/EK/11025). All experiments were done in accordance with recognized ethical principles in medical research involving human subjects, including the World Medical Association Declaration of Helsinki. Each participant provided signed informed consent.

Conflicts of interest: The authors declare no conflicts of interest.

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