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Metabolic Profiling of Saliva in Patients with Primary Sjögren’s syndrome

Jopi JW Mikkonen1, Maria Herrala1, Pasi Soininen2, Reijo Lappalainen1, Leo Tjäderhane3, Hubertus Seitsalo4, Raija Niemelä5, Tuula Salo6,7, Arja M Kullaa8,9 and Sami Myllymaa1

1SIB Labs and Department of Applied Physics, Faculty of Science and Forestry, University of Eastern Finland, Kuopio, Finland
2Department of Diagnostics and Oral Medicine, Institute of Dentistry, University of Oulu, Finland
3School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, Kuopio, Finland
4Department of Pedodontics, Cariology and Endodontology, Institute of Dentistry, Universities of Oulu and Turku, Finland
5Department of Oral Diseases, Oulu University Hospital, Oulu, Finland
6Dental Clinic Medone, Helsinki, Finland
7Department of Rheumatology, Oulu University Hospital, Oulu, Finland
8Institute of Dentistry, University of Helsinki, Helsinki, Finland
9Educational Dental Clinic, Health Centre of Oulu, Oulu, Finland

Abstract

Objective: To investigate the feasibility of 1H-NMR spectroscopy for metabolic profiling of human saliva samples and to determine whether the concentration of certain salivary metabolites, mainly representing small organic acids and amino acids, differ between patients with primary Sjögren’s syndrome (pSS) and healthy controls.

Methods: Stimulated whole-mouth saliva (SWMS) was collected from female pSS patients (n =15, all fulfilling the revised European Community proposed criteria). Salivary flow rate was immediately determined, samples were then frozen and subsequently analyzed by 1H-NMR spectroscopy in comparison with samples collected from healthy individuals (n=15).

Results: From each sample, up to 24 metabolites could be identified and quantified. Choline and taurine concentrations were very significantly higher in the pSS patients compared to healthy controls (p<0.001), but their concentrations correlated negatively with salivary flow rate. Alanine and glycine concentrations were significantly higher (p=0.004, p=0.007, respectively), whereas butyrate (p= 0.034), phenylalanine (p=0.026) and proline (p=0.032) were only slightly higher in pSS saliva samples than in controls.

Conclusions: NMR spectroscopy has a potential for quantitative metabolic profiling of saliva samples. NMR spectroscopy is suitable for the analysis of NAAs in saliva and it can bypass the other methods, which are normally suitable for analysis of just one metabolite.

Keywords: Saliva; Metabolomics; NMR spectroscopy; Amino acid neurotransmitters; Biomarkers; Sjögren’s syndrome

Introduction

Sjögren’s syndrome (SS) is an autoimmune rheumatic disease which causes chronic inflammation in the exocrine glands. Salivary glands’ hypofunction is a consequence of ducal and acinar cell destruction and causes lower salivary secretion [1]. Sjögren’s syndrome manifests in patients in two different forms: primary (pSS) and secondary (sSS). sSS usually is a consequence of some other rheumatic disease, for example systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA). pSS is otherwise already a disease by itself [2]. SS occurs worldwide with similar prevalence (0.5-1.5%) and around 90% of the patients are female. Typical age of the patients is between 40 and 60 years, but the disease also exists in all age groups [1]. Patients who suffer from SS have often a serious malfunction of exocrine glands, sometimes it is called the ‘sicca syndrome’. The main clinical features are xerostomia, ‘dry mouth’ and xerophthalmia, ‘dry eyes’ [3]. SS can affect also exocrine glands in esophagus, stomach, bowel, pancreas and bladder [4].

The pathogenesis of SS is complex and still partially unknown. Many factors, as genetic, hormonal, environmental, innate and adaptive immunity and the autonomic nervous system, have been thought to be involved in the pathogenesis of SS. The diagnostic criteria for SS has been presented by the American-European Consensus Group where the classification criteria includes the six main clinical findings; defining also separate diagnostic standards for pSS and sSS [5].

Recent advances in metabolic profiling techniques offer a powerful and promising approach to identify biomarkers associated with several disorders such as celiac disease [6,7], leukemia [8], breast cancer [9,10] and oral carcinomas [11]. A variety of different analytical techniques have been used in the metabolic profiling studies: currently the mass spectrometry (MS) has been utilized most frequently. Among MS techniques, especially two-dimensional gel electrophoresis (2D-GE) and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) have mostly been applied to analyze SS saliva samples [12-16].

Instead, another powerful metabolic profiling technique, namely nuclear magnetic resonance (NMR) spectroscopy, has remained largely underexplored in saliva analysis. Although some salivary metabolites have been successfully identified and inter- and intra-subject variability has been investigated by using 1H NMR [17-21] or 13C NMR spectroscopy [22], well-designed studies aiming to biomarker identification associated to certain health disorder are very rare [23].

*Corresponding author: Arja M Kulla, Department of Diagnostics and Oral Medicine, Institute of Dentistry, Faculty of Medicine, University of Oulu, Aapistie 3, SF-90220 Oulu, Finland; Tel: +358-40-1426319; E-mail: arja.kulla@oulu.fi

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The purpose of this study was to investigate the feasibility of $^1$H-NMR spectroscopy for metabolic profiling of human saliva samples and to clarify the potential of NMR spectroscopy for detecting the salivary metabolites associated with pSS. To the best of our knowledge, this study, for the first time, establishes the composition and concentration of metabolites in saliva samples collected from well-defined pSS patients and healthy control individuals.

Material and Methods

The pSS patients were around the Oulu area and had been diagnosed at the Department of Rheumatology, Oulu University Hospital, Oulu, Finland by RN. Detailed description of the patient inclusion and exclusion criteria has been presented previously by Niemelä et al. [24]. Briefly, all patients were required to fulfill the revised criteria of SS made by European Community [5]. Pregnant patients and patients with anticoagulant medication were left out. A total of 44 patients were screened, of which 22 participated in a study where saliva samples were collected [25]. The patients underwent an oral and dental examination. Exclusion criteria included smoking habits and oral or systemic diseases except pSS. Thus saliva samples of 15 female patients were available for the NMR analysis. Patients' ages varied between 28 and 68 years (mean age 48.6 years), and the time between the diagnosis of SS and the beginning of the study varied between 4 and 23 years. The control group contains 15 healthy, non-smoking female persons, age range 28 – 68 years (mean age 49.8 years). None of these subjects had any chronic disease or received any treatment regularly that could alter the salivary test results. The Oulu University Hospital Ethical Committee gave their approval for this study. All the patients gave their written consent according to the Declaration of Helsinki.

Collection of saliva samples

Saliva samples were collected using standard techniques according to Navazesh [26]. Briefly, stimulated whole-mouth saliva (SWMS) was collected in the morning (between 10 and 12 am) in order to assess any circadian effect. All subjects were asked to abstain from eating and drinking for at least 1 h before sample collection. Collection of stimulated saliva started with a stimulation time of 30 seconds by chewing paraffin wax (1 g; Orion Diagnostica, Espoo, Finland). Each subject was instructed to tilt their head forward, allow saliva to collect in the mouth, and let the saliva drain into a sterile 20 ml weighed polypropylene tube for 5 min. The saliva flow rate (ml/min) was measured immediately after saliva collection. Saliva samples were transported to the laboratory on ice and centrifuged at 3,000g for 20 min at 4°C. The supernatants were stored at -20°C for later use.

Sample preparation

450 µl of each saliva sample was thoroughly mixed with 50 µl of NMR-buffer (1.5 M KH₂PO₄, 2 mM NaN₃, 5.8 mM sodium-3-(trimethylsilyl) propionate-2,3,3-d₄, D₂O, pH 7.4) and subsequently centrifuged at 10,000×g for 5 min at +4°C to remove any solid debris. The resulting supernatant was transferred to 5 mm NMR tubes.

Data acquisition

The high-resolution 1D $^1$H were acquired using a Bruker AVANCE III HD spectrometer operating at 600.20 MHz ($^1$H observation frequency) and equipped with an inverse triple resonance Bruker CryoProbe Prodigy 5 mm probe head including ATM and Z-gradient coil. The spectrometer was controlled via TopSpin 3.2 (Bruker BioSpin GmbH) software. The samples were stored at +6°C in the sample changer until the measurement and were preheated to +25°C ca. 30 min prior to the measurement. Each sample was shimmed automatically using top shim routine. The 1D $^1$H data (64k data points) were recorded using a 1D T₂-relaxation-filtered pulse sequence) that suppress most of the broad macromolecule signals at +25°C with a 6.8 s repetition time (relaxation time 4.0 s and acquisition time 2.8 s) after 8 dummy scans using 96 transients with an automatically calibrated 90° pulse to achieve the required signal-to-noise level. A Bruker cpmg1d pulse sequence with $^1$H filter-time of 80 ms and irradiation field of 50 Hz to suppress the water peak was used. The 90° pulse was calibrated automatically for each sample. A constant receiver gain setting was used for all the samples.

Data processing

The acquired spectra were processed and phase corrected manually (TopSpin 3.0, Bruker BioSpin GmbH). Prior to the Fourier transformations to spectra, the measured free induction decays were multiplied with an exponential window function with a 1.0 Hz line broadening. The metabolites were identified by referring to the published data [22]. In total, 24 metabolites could be identified for quantification. Quantification of the metabolites was done using the constrained total-line-shape fitting type approach of the PERCH NMR software (PERCH Solutions Ltd, Kuopio, Finland), which allows an accurate quantification of identified metabolites even if the signals are overlapping or the baseline is not linear due to heavy protein background envelope [27]. The signal areas were referenced to an internal reference compound (TSP), which had a known concentration. The final concentrations are reported as µmol/l in saliva.

Statistical analysis

Metabolite levels are expressed as mean ± SD in tables and as box plots in figures.

The Shapiro-Wilk test and the values of kurtosis and skewness were used to analyze the data for normality distribution. Student’s t-test was used to compare stimulated salivary metabolite concentrations between pSS patient and healthy control groups.

Correlation were analyzed using Spearman’s test. SPSS software, version 19.0 (SPSS Inc., Chicago, IL, USA) was used in all analyses. Significance was set at P<0.05.

Results

Representative 1H-NMR spectra of saliva samples collected from a healthy participant and a pSS patient are shown in Figure 1. Up to 24 metabolites were assigned in each sample including organic acids (acetate, butyrate, citrate, formate, lactate, propionate, succinate), carbohydrates (butanol, ethanol, fucose, isoopropanol, galactose, glucose, methanol), amino acids (alanine, glycine, histidine, phenylalanine, taurine, tyrosine) as well as amines (choline, methylamine, proline, trimethylamine). Some metabolites, e.g. leucine, valine and isoleucine, were observed in only a few samples, and thus excluded from data analysis. The metabolite concentrations are presented in Table 1. The concentration of choline and taurine was significantly higher (p<0.001) in the pSS patients (n=15, all females, aged 28 to 68 years) compared to the healthy controls (n=15, all females, aged 28 to 68 years) (Figure 2). Moreover, alanine and glycine were significantly higher (p=0.004, p=0.007, respectively) in concentration in the pSS group. Butyrate (p=0.034), phenylalanine (p=0.026) and proline (p=0.032) were only slightly higher in SS saliva samples than in controls. Regarding other
metabolite concentrations, no statistically significant differences were found.

Finally we studied the associations between salivary flow rate and the concentration of metabolites. Salivary flow rate was significantly lower in the pSS group (0.82 ± 0.29 ml/min) than in the controls (1.99 ± 0.51 ml/min, p<0.001). The concentration of choline (r=-0.57, p=0.001) and taurine (r=-0.50, p=0.006) increases when the salivary flow rate decreases (Figure 3). Other metabolites did not significantly correlate with salivary flow rate.

Discussion

Saliva provides a unique window into the biological processes and functioning of the human body. It has been shown that saliva reflects the spectrum of health and disease states [28] and has therefore been labeled as “a mirror of the body” [29]. Most compounds found in blood are also present in saliva, but usually in lower concentrations [30]. Therefore, there have been concerns that the low concentrations of analytes in saliva may prevent salivary diagnostics from being clinically accurate enough. However, with the recent development of saliva collection methods, storage of samples and particularly new highly sensitive analysis techniques, low compound concentrations are no longer a limitation. NMR spectroscopy poses several advantages over classical biochemical assays. It represents a reproducible technique for investigating the salivary metabolites without extensive sample preparation, and the measurements are detectable in as little as 450 µl of saliva. Furthermore, NMR spectra provide information about physicochemical status of certain salivary metabolites.

Since the pSS is usually diagnosed only after advanced gland tissue
Table 1: Comparison of salivary metabolite concentrations between primary Sjögren’s syndrome (pSS) patients and healthy controls.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>pSS patients (n=15)</th>
<th>Controls (n=15)</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.D</td>
<td>Range</td>
<td>Mean ± S.D</td>
<td>Range</td>
</tr>
<tr>
<td>Acetate</td>
<td>1482.7 ± 952.6</td>
<td>202.7-3676.6</td>
<td>1503.0 ± 715.2</td>
<td>670.6-3035.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>23.7 ± 13.2</td>
<td>7.0-55.6</td>
<td>11.4 ± 7.3</td>
<td>2.5-27.6</td>
</tr>
<tr>
<td>Butanol</td>
<td>14.1 ± 12.1</td>
<td>1.5-45.1</td>
<td>8.6 ± 5.8</td>
<td>0.8-20.7</td>
</tr>
<tr>
<td>Butyrate</td>
<td>34.7 ± 29.0</td>
<td>7.2-107.8</td>
<td>16.4 ± 10.9</td>
<td>2.5-34.9</td>
</tr>
<tr>
<td>Choline</td>
<td>11.4 ± 5.3</td>
<td>4.5-24.7</td>
<td>4.1 ± 2.0</td>
<td>1.0-7.1</td>
</tr>
<tr>
<td>Citrate</td>
<td>18.3 ± 7.9</td>
<td>7.2-34.4</td>
<td>18.3 ± 13.8</td>
<td>3.4-46.5</td>
</tr>
<tr>
<td>Ethanol</td>
<td>25.4 ± 12.6</td>
<td>7.2-67.7</td>
<td>26.3 ± 59.6</td>
<td>7.9-241.3</td>
</tr>
<tr>
<td>Formate</td>
<td>152.0 ± 195</td>
<td>6.3-660.8</td>
<td>80.1 ± 63.3</td>
<td>3.3-220.5</td>
</tr>
<tr>
<td>Fucose</td>
<td>46.5 ± 35.9</td>
<td>3.7-113.6</td>
<td>66.3 ± 40.3</td>
<td>20.2-150.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>150.9 ± 120.5</td>
<td>16.0-387.7</td>
<td>51.5 ± 35.4</td>
<td>8.7-152.3</td>
</tr>
<tr>
<td>Histidine</td>
<td>30.3 ± 13.9</td>
<td>8.7-53.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isopropanol</td>
<td>1.84 ± 0.8</td>
<td>0.2-3.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>265.6 ± 140.4</td>
<td>25.4-509.7</td>
<td>198.3 ± 174.5</td>
<td>14.1-649.0</td>
</tr>
<tr>
<td>Methanol</td>
<td>28.5 ± 17.5</td>
<td>9.7-65.6</td>
<td>27.7 ± 9.2</td>
<td>8.3-43.9</td>
</tr>
<tr>
<td>Methylamine</td>
<td>2.1 ± 1.7</td>
<td>0.5-5.7</td>
<td>2.0 ± 0.9</td>
<td>1.1-3.8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>14.3 ± 8.4</td>
<td>2.6-28.6</td>
<td>8.5 ± 3.6</td>
<td>2.6-16.8</td>
</tr>
<tr>
<td>Proline</td>
<td>133.5 ± 114.5</td>
<td>9.1-362.9</td>
<td>59.1 ± 44.1</td>
<td>17.8-160.5</td>
</tr>
<tr>
<td>Propionate</td>
<td>284.3 ± 205.7</td>
<td>49.8-722.1</td>
<td>235.7 ± 156.2</td>
<td>69.0-621.9</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>17.6 ± 11.2</td>
<td>0.9-37.5</td>
<td>10.4 ± 7.8</td>
<td>0.0-24.3</td>
</tr>
<tr>
<td>Succinate</td>
<td>33.8 ± 32.1</td>
<td>6.7-131.8</td>
<td>28.2 ± 15.7</td>
<td>5.3-49.5</td>
</tr>
<tr>
<td>Taurine</td>
<td>110.5 ± 49.6</td>
<td>18.6-213.0</td>
<td>48.3 ± 29.0</td>
<td>12.5-111.5</td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>1.4 ± 1.4</td>
<td>0.1-4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>26.7 ± 18.5</td>
<td>5.1-75.4</td>
<td>19.6 ± 11.4</td>
<td>6.4-50.5</td>
</tr>
<tr>
<td>1,2-propanediol</td>
<td>40.1 ± 71.4</td>
<td>9.2-290.0</td>
<td>27.3 ± 14.2</td>
<td>9.8-59.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Student's t-test; p< 0.001<sup>***</sup>; p<0.01<sup>**</sup>; p<0.05<sup>*</sup>

Figure 3: The correlations between the salivary flow rate and concentration of choline and taurine. pSS patients (n=15) are marked by open circles and healthy controls (n=15) by black squares. The solid line represents the linear regression curve of the best fit. r = Spearman’s correlation coefficient.
these metabolites are among the amino acids with important roles as neurotransmitters, which have been the focus of increasingly intense in biomedical research in recent years. Furthermore, these metabolites are associated to muscarinic-M3 receptors which play a key role in regulating salivary flow [33]. Previously, it has been suggested that deficient neural regulation of the secretory process might exist [34]. This study demonstrated that concentrations of certain amino acids, i.e. choline and taurine in saliva are strongly associated with changes in salivary flow.

Many methods have been developed for the detection of amino acids [35-37], but in this study we showed that the neurotransmitter amino acids (NAAs) can be identified in saliva with NMR method. However the role of NAAs in saliva is still unknown, although changes in NAA levels in other biological fluids may correlate with a number of neurological diseases such as Alzheimer’s [38] and Parkinson’s diseases [39]. The NMR metabolomics can contribute to better understanding of the molecular mechanism of Sjögren’s syndrome. Detection of neurotransmitters and other metabolites in saliva may add information on the pathogenesis of SS and neural destruction of salivary glands.

This study suggests that it is possible to measure the levels of disease-specific metabolic components of saliva using 1H-NMR spectroscopy. In the diagnostics based on salivary metabolites, it is clear that a combination pattern of several biomarkers but not a single one may define a specific disease [29]. Specific biomarkers for SS could possibly provide preindication of the syndrome, and be used to monitor the severity of the disease. This promising potential of analyzing the level of specific salivary metabolites has given us a clearer idea to study the role of salivary NAAs in the oral defense mechanism. However, further comparisons should be made between SS and other (autoimmune) disorders to reveal and refine the validity of these metabolic biomarkers for the diagnostics of SS.

Despite significant advantages, there are also some limitations in use of NMR spectroscopy as a screening tool. A modern NMR spectrometer uses superconducting magnets that must be cooled with liquid helium making them very complex and expensive machines to purchase and operate. In addition, performing measurements and interpretation of acquired spectra are somewhat challenging and time-consuming. Also relatively large saliva sample volume is typically needed before and after pilocarpine treatment. However, future technology development (e.g. picoSpin™ miniature NMR spectrometer, Thermo Scientific) combined with more robust data analysis tools will help to overcome present limitations.

In conclusion, our data suggest that changed concentration of certain metabolites detected in SS saliva reflects the functional impairment of the secretory processes observed in this syndrome. The results presented here should be tested in larger patient cohorts in order to reveal if the NMR spectroscopy technique suits for noninvasive screening and monitoring of the severity of SS. Due to the findings of this study, NMR spectroscopy is suitable for the analysis of NAAs in saliva and it can bypass the other methods, which are normally suitable for analysis of just one metabolite.

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References


