SALIVARY METABOLOMIC PROFILING FOR DISCRIMINATING PRIMARY SJOGREN SYNDROME FROM HEALTHY CONTROLS: IDENTIFYING POTENTIAL BIOMARKERS - A PILOT STUDY

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ORIGINAL LITERARY WORK DECLARATION

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Field of Study: Applied Oral Pathology

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SALIVARY METABOLOMIC PROFILING FOR DISCRIMINATING PRIMARY SJOGREN SYNDROME FROM HEALTHY CONTROLS: IDENTIFYING POTENTIAL BIOMARKERS- A PILOT STUDY

ABSTRACT

Background: Salivary biomarkers such as proteins, metabolites, hormones, and nucleic acid can provide biological information for a variety of medical problems such as cancer, stress, systemic disorders, and neurodegenerative and infectious diseases. The proteome and metabolomic alterations observed in saliva appear to match those observed in blood, reflecting the cells' cellular activity and physiological status. **Objectives:** This study aims to identify the potential salivary biomarkers that can help distinguish patients with primary Sjogren syndrome (pSS) from healthy controls. **Methods:** A case-control study of the salivary metabolome of two different groups of subjects; 5 control cases, and 5 pSS patients. Salivary metabolites profiling was carried out using 2 analytical approaches: Nuclear magnetic resonance (^{1}H-NMR) and Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (LC-Q-TOF-MS) with HMDB, BRMB, and Metlin databases respectively. Metabolite biomarkers identified will be further interpreted through chemometric analysis (unscrambler software) and statistical packages for the social sciences (SPSS) software to evaluate the diagnostic ability of each of the discriminant metabolites. **Results:** A total of 269 metabolites were obtained using LC-Q-TOF-MS, there was one metabolite that was associated with pSS which is purine. Purine was significantly upregulated in the saliva of pSS when compared to control (p-value = 0.01). For ^{1}H-NMR, a total of 241 metabolites were found with only 63 metabolites with VIP scores >1. The top 5 upregulated metabolites were mapped into 12 metabolic pathways. **Conclusion:** There was a significant difference in the salivary metabolites profile (purine) between patients with pSS and healthy control subjects thus the study hypothesis was accepted, and the null hypothesis was rejected.

Keywords: Saliva biomarkers, primary Sjogren syndrome, metabolomics, liquid chromatography-quadrupole time-of-flight mass spectrometry, nuclear magnetic resonance.

PROFIL METABOLOMIK AIR LIUR UNTUK MEMBEZAKAN SINDROM SJOGREN PRIMER DARI KAWALAN JANGKITAN SIHAT: MENGENALPASTI BIOMARKER BERPOTENSI – KAJIAN PERINTIS

ABSTRAK

Latar Belakang: Biomarker air liur seperti protein, metabolit, hormon, dan asid nukleik boleh menyediakan maklumat biologi untuk pelbagai masalah perubatan seperti kanser, stres, gangguan sistemik, dan penyakit neurodegeneratif dan berjangkit. Perubahan proteoma dan metabolomi yang diamati dalam air liur kelihatan sesuai dengan yang diamalkan dalam darah, mencerminkan aktiviti sel dan status fisiologi sel. **Objektif**: Kajian ini bertujuan untuk mengenal pasti biomarker air liur yang berpotensi yang boleh membantu membezakan pesakit dengan sindrom Sjogren primer (pSS) daripada kawalan yang sihat (HCs). **Kaedah**: Satu kajian kajian kawalan kes metaboloma salivari dua kumpulan subjek yang berbeza; 5 HCs, dan 5 pesakit pSS menggunakan dua jenis sampel yang tidak distimulasi air liur (AS) dan cuci mulut (OR). Profil metabolit salivary dijalankan menggunakan 2 pendekatan analitik: resonansi magnet nuklear (1H-NMR) dan Kromatografi cecair Quadrupole Time-of-Flight Mass Spectrometry (LC-Q-TOF-MS) dengan HMDB, BRMB, dan Metlin pangkalan data masing-masing. Biomarker metabolit yang diidentifikasi akan diinterpretasikan lebih lanjut melalui analisis kemometri (perisian uncrambler) dan perisian statistik untuk sains sosial (SPSS) untuk menilai keupayaan diagnostik masing-masing metabolit diskriminatif. **Hasil**: Sebanyak 269 metabolit diperolehi menggunakan LC-Q-TOF-MS, terdapat satu metabolit yang dikaitkan dengan pSS iaitu purin. Purine telah ditingkatkan secara signifikan dalam air liur pSS berbanding dengan kawalan (nilai p = 0.01). Untuk 1H-NMR, jumlah 241 metabolit ditemui dalam hanya 63 metabolit dengan skor VIP > 1. Lima metabolit teratas yang diupregulasikan telah dipetakan ke dalam 12 laluan metabolik **Kesimpulan**: Terdapat perbezaan yang signifikan dalam profil metabolit salivari (purine) antara pesakit

dengan pSS dan subjek kawalan yang sihat, oleh itu hipotesis kajian diterima, dan hypothesis nol ditolak.

Kata Kunci: Biomarker air liur, sindrom Sjogren primer, metabolomik, kromatografi cecair-spektrometri jisim kuadrupol masa-penerbangan, resonans magnet nuclear.

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Lastly, for my late mother, Rohizah bt Haji Shaari, this is my promise to you, ma.

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LIST OF SYMBOLS AND ABBREVIATIONS

6PPC	: 6-point pocket chart
ACR/EULAR	:American College of Rheumatology European League Against Rheumatism
COPD	: Chronic Obstructive Lung Disease
CTD	: Connective Tissue Disease
DNA	: Deoxyribonucleic acid
DHA	: Docosahexaenoic
EPA	: Eicosapentaenoic
FID	: free induction decay
GC-MS	: Gas Chromatography-Mass Spectrometry
GSG	: Glutathione
GSGH	: Oxidized glutathione
HCs	: Healthy Controls
HCV	: Hepatitis C virus
HIV	: Human Immunodeficiency Virus
HPLC	: High-Performance Liquid Chromatography
ICDAS	: International Caries Detection and Assessment System
IFN	: Interferon rheumatoid factor (RF),
LC-Q-ToF-MS:	: Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry
LMW	: low molecular weight
MHC	: major histocompatibility complex
mRNAs	: Messenger ribonucleic acid
MS	: Mass Spectrometry
NHL	: Non-Hodgkin's lymphoma
NMR	: Nuclear Magnetic Resonance
NS-C	: non-Sjogren’s disease participants
OPLS-DA	: Orthogonal Projections to Latent Structures Discriminant Analysis
OR	: Oral Rinse
PCA	: Principal Component Analysis
pSS	: primary Sjogren’s syndrome
RA	: Rheumatoid Arthritis
RF	: Rheumatoid factor
SS	: Sjogren syndrome
sSS	: secondary Sjogren’s syndrome
UPLC/MS	: Ultra-Pure liquid chromatography-mass spectrometry
VIP	: Variable Importance in Projection

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CHAPTER 1: INTRODUCTION

1.1 Study Background

Sjogren syndrome (SS) is a chronic inflammatory autoimmune disease that is due to mononuclear cell infiltration of exocrine glands, notably the lacrimal and salivary glands, although other exocrine glands may also be affected (Jonsson et al., 2018). There are two forms of SS which are as an entity by itself, primary Sjogren syndrome (pSS) with symptoms like dry mouth and dry eyes in the absence of connective tissue disease. Another form is associated with other autoimmune diseases, which is called secondary Sjogren syndrome (Goules & Tzioufas, 2016).

The SS has a strong female predilection with 9:1 female: male ratio (Chevet et al., 2023) and is more prevalent in Caucasian women, with the mean age of onset usually in the fourth to fifth decade (Maciel et al., 2017).

Changes in salivary gland pathology are thought to be reflected in the quantity and quality of saliva, which are characteristic features of pSS (Kageyama et al., 2015). Typically, clinical symptoms commonly shared by most SS such as severe dry mouth and eyes, pain, and disabling fatigue end up reducing the quality of life resulting in anxiety and depression (Alt-Holland et al., 2023). Early pSS diagnosis and hence, early treatment for mucosal symptoms and dryness are critical. This will assist in avoiding consequences brought on by the disease's advancement.

There is currently no single clinical, laboratory, pathological, or radiological feature that can be regarded as the definitive standard for diagnosing or classifying this illness The diagnostic criteria for pSS syndrome are outlined by the 2016 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) Consensus

Classification Criteria. These criteria are based on the total of weighted scores assigned to five factors: the presence of anti-SSA/Ro antibodies, focal lymphocytic sialadenitis with a focus score of at least 1 focus per square millimeter, an ocular staining score of 5 or higher (or a van Bijsterveld score of 4 or more) in at least one eye, a Schirmer's test result of 5 mm or less in 5 minutes, and an unstimulated salivary flow rate (SFR) of 0.1 mL per minute or less (Shiboski et al., 2017).

Saliva is a clear, complicated biofluid made up of several different components, such as proteins, messenger ribonucleic acids (mRNAs), deoxyribonucleic acids (DNAs), metabolites, enzymes, hormones, growth factors, antimicrobial components, and antibodies (Zhang et al., 2016). It is well known that the composition of saliva will dramatically change in response to different physiological stimuli, stressors, or injuries (Dame et al., 2015). The proteome and metabolomic alterations observed in saliva (particularly parotid-derived saliva) appear to match those observed in blood (Chiappin et al., 2007; Takeda et al., 2009). Research data indicates that variations in these substances' concentrations can reflect both local and oral disorders as well as distal pathological changes and are linked to the aetiology of several diseases.

Unlike blood, saliva collection is a straightforward, non-invasive, reproducible, and inexpensive process. New developments in technology have made it possible to analyse saliva and track the health of several physiological systems (Zhang et al., 2016). Utilizing recently developed "omics" technology, saliva has been studied to find potential biomarkers for pSS diagnosis and prognosis (Alt-Holland et al., 2023).

The term "metabolome" describes all the small-molecule metabolites that may be identified in a biological sample. The identification and validation of metabolic markers

of disease can happen more rapidly due to advances in metabolomics technologies. Techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the primary analytical methods used in metabolomics. In a single measurement, both techniques yield data on the relative and absolute concentrations of various groups of metabolites (Zheng et al., 2020).

Differential metabolomic expression in the saliva of pSS patients and healthy control (HCs) subjects has been observed in several studies (Alt-Holland et al., 2023; Herrala et al., 2021; Kageyama et al., 2015; Li et al., 2022; Mikkonen, 2012; Setti et al., 2023) which may help establish diagnostic biomarkers.

1.2 Rationale and Significance

Salivary biomarkers may be useful adjunct diagnostic tools for the detection of pSS. Identification of the differences in the salivary metabolites profile between pSS patients and HCs has the potential for non-invasive screening and as an adjunct diagnostic tool to discriminate pSS from HCs.

1.3 Research questions

- Are there any similarities between the salivary metabolites of unstimulated saliva (US) samples and oral rinse (OR) samples?
- What are the salivary metabolomes in HCs and pSS patients?
- Can the differences in the salivary metabolomes between these two groups (HCs and pSS) be used as a diagnostic tool to differentiate pSS from HCs?

1.4 Aim

To identify the potential salivary biomarkers that can help in distinguishing patients with pSS from HCs.

1.5 Specific objectives

- To determine the differences between the salivary metabolites of US samples and OR samples.
- To determine the various salivary metabolites in HCs and in patients with pSS.
- To investigate the potential utility of salivary metabolome differences as a diagnostic tool for effectively distinguishing pSS patients from HCs.

1.6 Study Hypothesis

There are statistically significant differences in the salivary metabolomes between patients with pSS and HCs.

1.6.1 Null Hypothesis

There are no statistically significant differences in the salivary metabolomes between patients with pSS and HCs.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The original description of SS is widely attributed to Jan Mikulicz-Radecki. He reported on a 42-year-old man who had acinar atrophy, round-cell infiltration, and enlargement of the parotid and lacrimal glands in 1892. The main pathological lesion seen in SS is focal lymphocytic sialadenitis. Because it did not offer enough prognostic or therapeutic information, the name Mikulicz's syndrome—which could refer to a wide range of conditions—fell into disuse. These conditions included lymphoma, sarcoidosis, tuberculosis, and other diseases. Nevertheless, when lymphocytic infiltrates are seen on salivary gland biopsies, the phrase "Mikulicz's syndrome" is occasionally used to characterize the condition.

In the early 1900s, Swedish doctor Henrik Sjögren initially documented a group of ladies with dry mouths and eyes and persistent arthritis. Sjögren first used the term keratoconjunctivitis sicca to differentiate this disease from dry eyes resulting from a vitamin A deficiency (xerophthalmia). Rheumatologists now have a better understanding of the SS and, most importantly for patients, can counsel them on how to manage it.

The majority of those affected with pSS, a chronic systemic autoimmune illness, are women going through menopause. The main clinical presentation of pSS is dry mouth and eyes, which are caused by the infiltration of periductal lymphocytic cells in the lacrimal and salivary glands. Activation of type I and II interferon (IFN) pathways, rheumatoid factor (RF), serum autoantibody over-secretion (anti-Ro/SSA, anti-La/SSB), and B-cell hyperactivity are recognized clinical markers. The development of non-Hodgkin's lymphoma (NHL), the most serious side effect of SS, affects 5–10% of pSS patients and is the main cause of SS-related mortality. NHL has an estimated relative risk of 4–40 times higher than the general population (Skarlis et al., 2022).

Given the wide spectrum of clinical manifestations of SS due to the presence of variation in extra glandular manifestations, disease diagnosis can often be challenging. New developments in technology have made it possible to analyze saliva, utilizing recently developed "omics" technology, to find potential biomarkers for pSS diagnosis and prognosis (Alt-Holland et al., 2023).

2.2 Sjogren Syndrome

2.2.1 Definition

Sjogren's syndrome (SS) is a connective tissue disease characterized primarily by dryness of the oral mucosa and eyes, which are the most common and recognizable symptoms. The disease manifests in two forms: primary Sjogren's syndrome (pSS) and secondary Sjogren's syndrome (sSS). Primary SS is an auto-immune illness that causes lymphocytic infiltration of the salivary and lachrymal glands, resulting in xerostomia and xerophthalmia in the absence of any connective tissue disease (CTD) (Bloch et al., 1992). Additionally, 30-50% of patients with pSS experience systemic manifestations such as cutaneous, extra-glandular, and pulmonary involvement (Rischmueller et al., 2016). Secondary SS is diagnosed when symptoms of SS are present with other systemic connective tissue diseases. Secondary SS is often identified based on symptoms of dryness that occur alongside another connective tissue disease such as systemic lupus erythematosus (22%), rheumatoid arthritis (53%), or scleroderma (Sebastian et al., 2019).

2.2.2 Epidemiology

Primary SS is far from a rare condition, with an incidence range of 3.5–3.9 per 100,000 person-years or 0.3–26.1 per 100,000 persons (Thurtle et al., 2024). This illness can affect people of all ages, but women, particularly those in older age groups, exhibit the highest incidence and prevalence. The condition commonly appears in middle-aged people, but it can also affect youngsters and the elderly (Alani et al., 2018). Women are affected 6 to

9 times more frequently than men (Chevet et al., 2023). The age of symptom onset ranges from 34 to 57 years, while the age at diagnosis typically falls between 40 and 67 years (Thurtle et al., 2024). The incidence pSS was highest in the Asian population, followed by the Whites, with the lowest incidence rates reported among Latinos and Blacks (Beydon et al., 2023). Approximately half of patients have rheumatoid arthritis or other connective tissue illnesses, such as lupus. Mortality in pSS appears to be higher mainly in patients with extra-glandular manifestations, pulmonary involvement, and non-Hodgkin lymphoma (Beydon et al., 2023).

2.2.3 Aetiology

The pathophysiology of SS involves a concurrent disruption of both the innate and adaptive immune systems, encompassing poorly understood humoral and cell-mediated mechanisms. While these disorders are recognized globally, a genetic predisposition is believed to play a role, particularly involving the major histocompatibility complex (MHC) locus. The DR2 and DR3 alleles at the DRB1 locus are the major histocompatibility complex (MHC) genes most closely linked to primary Sjogren's syndrome (pSS). The genes HLA-DQB1 and HLA-DQA1 have a strong connection. Individuals sharing haplotypes in the HLA-DQA/DQB region have an increased relative risk (Matzaraki et al., 2017). It is hypothesized that these major histocompatibility haplotypes when combined with specific environmental stimuli, may cause an abnormal immunological response.

2.2.4 Diagnostic criteria

The syndrome's heterogeneous presentation poses a significant barrier to improving diagnosis and treatment. Hence, a worldwide set of criteria for pSS has been devised and validated, making it suitable for clinical trials. Diagnostic criteria are provided in the 2016 ACR/EULAR Consensus of Classification Criteria for pSS which are based on the sum

of weighted scores applied to five items as listed in Table 2.1. People who fit the criteria for pSS are those who have a total score of ≥ 4 in the categories above and exhibit signs or symptoms suggestive of SS. These criteria define fundamental features of the illness and can serve as a common language for the diagnosis and prognosis of the disease, as well as for future scientific communication, information exchange, and collaborative studies.

Table 2.1 2016 (ACR)/(EULAR) Classification Criteria for primary Sjogren syndrome

Criteria	Score
Anti-Ssa (Ro) Antibody Positivity	3
Focal lymphocytic sialadenitis with a focus score ≥ 1foci/mm^2	3
Ocular staining score ≥ 5 (or van Bijsterveld score ≥ 4)	1
Schirmer test ≤ 5 mm/5 minute	1
Unstimulated salivary flow rate ≤ 0.1 mL/minute	1

2.2.5 Biomarkers in primary Sjogren Syndrome

Identifying biomarkers is crucial for it is not just as a quantitative measure to allow for a precise diagnosis but can also be used for assessing a disease process as well as monitoring response. Biomarkers can be defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention to treatment (Biomarkers Definitions Working Group., 2001). There are several multiple biomarkers for SS. The most visible markers are found in serum (e.g. autoantibodies and cytokines), DNA (by gene profiling and genome-wide association studies), and cells (based on their phenotypes and functions). Recently, saliva has been studied to find potential biomarkers for pSS diagnosis and prognosis as it appears to match those observed in blood (Chiappin et al., 2007).

2.3 Saliva

2.3.1 Saliva composition

Saliva contains many electrolytes, including sodium, potassium, calcium, magnesium, bicarbonate, and phosphates. It also contains immunoglobulins, proteins, enzymes, mucins, and nitrogenous products (urea and ammonia). These components perform related functions in the following general areas: Saliva's pH and buffering capacity are influenced by bicarbonates, phosphates, and urea, while macromolecule proteins and mucins contribute to plaque metabolism. Calcium, phosphate, and proteins work together to prevent demineralization and remineralization, and immunoglobulins, proteins, and enzymes provide antibacterial action (Humphrey & Williamson, 2001).

2.3.2 Salivary gland

There are three pairs of major salivary glands. The parotid gland is the largest of the three main salivary glands. It runs between the sternocleidomastoid muscle and the masseter, from the mastoid tip to just below the angle of the mandible. A short tail extends from the gland's inferior margin, separated from the submandibular area only by the stylomandibular ligament. The excretory duct of the parotid gland is Stensen's duct, which opens into the oral cavity at the level of the buccal mucosa of the second maxillary molar. It secretes mainly serous saliva, a watery solution rich in enzymes (Carlson, 2000).

The submandibular gland, the second-largest gland, weighs about half as much as the parotid and is located inferior to the mandible, between the anterior and posterior bellies of the digastric muscle. The primary excretory duct, also known as Wharton's duct, emerges from the smaller, deep lobe inferior to the mucosa of the mouth floor, enters the oral cavity, and opens into the frenulum linguae. The hypoglossal nerve runs parallel and inferior to Wharton’s duct (Carlson, 2000).

The sublingual glands are located beneath the mucosa of the mouth's floor and above the mylohyoid muscle (between the mandible and genioglossus). The submandibular duct and sublingual nerve are in the middle, between the base of the tongue and the sublingual gland. Rather than having one main duct, it has a series of short ducts that project straight into the floor of the mouth, the ducts of Rivinus, and a common duct, known as Bartholin's duct, which joins with the submandibular gland's duct at the sublingual caruncula (Carlson, 2000).

2.3.3 Salivary flow rates

The parotid, submandibular, and sublingual salivary glands produce 90% of saliva, with minor salivary glands accounting for the remaining 10%. Whole saliva refers to the amount secreted by both the major and minor glands. Saliva contains 99% water and 1% protein, electrolytes, and other substances. Salivary flow rates vary greatly among individuals. The average daily saliva output is between 0.5 and 1.5L. The US flow rate is around 0.3-0.4 mL/minute. After sleep, the rate lowers to 0.1 mL/minute. However, after eating, chewing, and other stimulating activities, it climbs to 4.0-5.0 mL/minutes (Iorgulescu, 2009). Anything above 0.1 mL/minute is considered normal flow in unstimulated saliva. Any unstimulated flow rate below 0.1 mL/minute is considered hypofunction. The autonomous nerve system primarily regulates salivary gland secretion. Parasympathetic stimulation creates watery saliva, while sympathetic stimulation stimulates viscous saliva (Pedersen et al., 2005).

The quantity and quality of saliva are affected by underlying systemic disease and drugs. Hyposecretion and hypersecretion of saliva flow rate are known as xerostomia and sialorrhea, respectively (Iorgulescu, 2009). Saliva alterations can indicate exposure to chemicals and infections, as well as assess sensitivity to certain illnesses.

2.4 Metabolomics

2.4.1 Metabolomics profiling

Metabolomics, or metabolite profiling, is the quantitative study of endogenous and exogenous small molecules from a biological system. Metabolite profiling can be approached using one of two analytical techniques. The first approach, which is targeted metabolomics (e.g. MS) uses specialized methods to quantify specific chemicals. This technique is ideal for research topics that involve measuring a small number of analytes. Targeted metabolomics can be applied to quantitate less than a few dozen compounds, but some workflows have been developed to monitor several hundred metabolites (Nikolskiy et al., 2015). At the other end, is untargeted metabolomics like NMR which involves measuring all small molecules in a sample. Untargeted metabolomics aims for broad and unbiased analysis; however, the physiochemical diversity of the metabolome restricts the number of substances examined in a single experiment (Patti, 2011). The advantages and disadvantages of MS and NMR techniques are summarized in Table 2.2.

Table 2.2 Advantages and disadvantages of MS and NMR techniques

	Nuclear magnetic resonance (NMR)	Mass spectrometry (MS)
Sensitivity	Low	High
Reproducibility	Very high	Average
Number of detectable metabolites	30-100	300-1000+ (depending on whether GC-MS or LC-MS is used)
Targeted Analysis	Not optimal for targeted analysis	Better for targeted analysis than NMR
Sample preparation	Minimal sample preparation required	More complex sample preparation required
Tissue extraction	Not required – tissues can be analyzed directly	Requires tissue extraction
Sample analysis time	Fast – the entire sample can be analyzed in one measurement	Longer than NMR – requires different chromatography techniques depending on the metabolites analyzed
Instrument Cost	More expensive and occupies more space than MS	Cheaper and occupies less space than NMR
Sample Cost	Low cost per sample	High cost per sample

2.4.1.1 Liquid chromatography Quadrupole time-of-flight- mass spectrometry

Liquid chromatography Quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS) is an advanced analytical technique combining the separation capabilities of liquid chromatography (LC) with the highly accurate mass analysis capabilities of quadrupole time-of-flight mass spectrometry (QTOF MS).

In this method, complex mixtures are first separated into individual components by LC, which utilizes a liquid mobile phase to pass the sample through a column packed with a stationary phase. The separated compounds are then ionized and introduced into the mass spectrometer. The quadrupole acts as a mass filter, selecting ions based on their mass-to-charge ratio (m/z), which are then analyzed by the TOF detector. The TOF

analyzer measures the time it takes for ions to travel a known distance, allowing for precise determination of their m/z ratios.

Recent liquid chromatography generally uses very small particles packed and operating at relatively high pressure and is referred to as HPLC; modern LC/MS methods use HPLC instrumentation essentially exclusively for samples. The underlying principle in HPLC is adsorption. In HPLC, a liquid at high pressure (the mobile phase) forces the sample through a column packed with a stationary phase, which is often made up of irregularly or spherically shaped particles selected or derivatized to achieve specific types of separations (Ramachandram & Dinesh, 2016).

Mass spectrometry (MS) is an analytical technique for determining the mass-to-charge ratio of charged particles. It is used to determine particle masses, and the elemental content of a sample or molecule, and to understand the chemical structures of molecules like peptides and other chemical compounds. In a usual MS operation, a sample is put onto the device and vaporized. The components of the sample are ionized using one of several techniques (for example, by striking them with an electron beam), resulting in the creation of charged particles (ions). In an analyzer, electromagnetic fields separate ions based on their mass-to-charge ratio. The ions are usually discovered using a quantitative approach. The ion signal is converted into mass spectra (Ramachandram & Dinesh, 2016).

2.4.1.2 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry can analyze hundreds or thousands of metabolites in a single run. Although Isidor Rabi is generally credited with the Nobel Prize in Physics in 1944 for the discovery of NMR, he did so in an inherently "unnatural" context. In the late 1940s and early 1950s, NMR spectroscopy was created independently by the Purcell group at Harvard University and the Bloch group at Stanford University. Edward Mills Purcell and Felix Bloch received the 1952

Nobel Prize in Physics for their inventions (Partain, 2004). Since then, numerous studies have focused on using NMR spectroscopy for metabolic profiling and, when paired with multivariate analysis, can be utilized as a biomarker diagnostic tool.

2.4.1.3 Gas chromatography/mass spectrometry

Since the 1980s, plant metabolite profiling has relied on Gas chromatography/mass spectrometry (GC/MS) -based metabolomics approaches. Metabolomics technology has gained popularity in recent years for toxicity, biomarker development, illness diagnosis, and classification in both animals and humans (Fancy & Rumpel, 2008). Gas chromatography (GC) is ideal for analyzing hydrophobic compounds produced by organic chemists or extracted from natural sources with organic solvents. It differs from NMR and LC-MS in its ability to analyze a wide variety of nonpolar analytes while LC-MS and NMR target the polar molecules. The overlap between GC-MS and LC-MS metabolomic profiling data is restricted due to distinct separation and ionization methods (Fancy & Rumpel, 2008).

2.4.2 Plasma metabolomics

Plasma untargeted metabolomics is a standard for assessing the mechanisms behind human diseases and identifying novel biomarkers as it offers an environment for the transfer of hydrophobic compounds. Plasma metabolomics has better reproducibility than in serum, but higher metabolite concentrations were found in serum making it more sensitive for biomarkers detection (Yu et al., 2011). Plasma metabolomic approaches have been widely used in determining new disease biomarkers such as acute ischemic stroke (Zhai et al., 2019), tuberculosis meningitis (Huynh et al., 2022), type 2 diabetes mellitus (Doumatey et al., 2024), and breast cancer (Wei et al., 2021).This is a promising trend in using plasma in diagnosing these diseases. Fernández-Ochoa et al. (2020) found 31 plasma metabolites that showed significant differences between healthy volunteers

and pSS patients and were proposed for identification. Of these, 12 plasma metabolites could be annotated. Docosahexaenoic (DHA), eicosapentaenoic (EPA), linolenic, and oleic acids appeared upregulated in plasma samples from pSS patients.

2.4.3 Urinary metabolomics

Urine contains a diverse spectrum of metabolites and is mostly made of water, chemical molecules, electrolytes, and metabolites. This composition closely mirrors the metabolic processes that take place throughout the body. Urine is essential for disease diagnosis and biomarker screening in metabolomics due to its non-viscous, non-invasive nature, and lower protein and fat concentration than other bodily fluids. Urine metabolomics has been used to discover potential biomarkers for cancer diagnosis, especially bladder cancer (Wang et al., 2022), lung function in Chronic obstructive pulmonary disease (COPD) (McClay et al., 2010), and detection of jaundice syndrome in patients with liver disease (Wang et al., 2012). Although most of this research requires replication and validation, it managed to demonstrate the ability of urine metabolomics as a screening tool for identifying novel biomarkers. Fernández-Ochoa et al. (2020) discovered 38 urine metabolites showed significant differences between healthy volunteers and pSS patients and were proposed for identification. Of these, 24 urinary metabolites could be annotated. One of the most notable findings has been the number of metabolites derived from amino acids found to be deregulated in urine samples, many of which are dipeptides such as histidine-hydroxyproline, leucyl-proline, valyl-proline, phenylalanine, gamma-glutamyl valine, and gamma-glutamyl isoleucine.

2.4.4 Salivary metabolomics

According to Dame et al. (2015) there is a collection of 853 low molecular weight (LMW) metabolites which can represent both healthy and pathologic conditions in the human body. These metabolites are the substrates, intermediates, and end products of

biochemical reactions. Many of these metabolites enter the saliva via the blood via transcellular, intracellular, paracellular, or extracellular routes involving passive diffusion or active transport inside the salivary glands and gingival sulcus (Spielmann & Wong, 2011).

Several studies have previously been published suggesting that saliva can be utilized to diagnose periodontal disorders, premature birth, mouth cancer, lung cancer in the early stages, pancreatic cancer, breast cancer, viral infections, bacterial infections (Helicobacter pylori), cardiovascular disease, and diabetes (Cámpora et al., 2003; Fidalgo et al., 2013; Kaufman & Lamster, 2002; Spielmann & Wong, 2011).

Low molecular weight (LMW) metabolites can be measured using metabolomics, and current metabolomics technologies facilitate the rapid discovery and validation of metabolic indicators of disease. Techniques used in metabolomics, such as LC/MS or NMR can routinely measure tens to hundreds of metabolites with excellent precision and are suitable for discovery studies in human cohorts.

2.4.4.1 Current data on salivary metabolomics

Some researchers have advocated for the use of saliva in clinical diagnostics (Wong, 2006). For example, saliva testing is now the most used approach for diagnosing Cushing's syndrome and Addison's illness. For over 2000 years, saliva-based diagnostics (using taste, viscosity, or fragrance) have been utilized in traditional or folk medicines (Wei et al., 2011).

2.4.4.2 Salivary metabolomics and primary Sjogren syndrome

Mikkonen et al. (2012) detected 24 metabolites in stimulated entire saliva using untargeted ^{1}H-NMR, with significant differences in particular compounds between analysis groups. The concentrations of choline, taurine, alanine, and glycine in pSS

patients were substantially greater than in HCs. On the other hand, butyrate, phenylalanine, and proline showed relatively minor differences in pSS saliva samples compared to HCs.

Kageyama et al. (2015) examined the metabolite profiles of 14 pSS patients with GC-MS. Of the 88 metabolites examined, 41 were considerably lower in pSS patients compared to HCs, most likely due to salivary gland injury; glycine, tyrosine, uric acid, and fucose contributed to the loss of biodiversity in the pSS in comparison to HCs salivary samples.

Herrala et al. (2021) discovered 24 metabolites in salivary samples using 1H-NMR. A second study was carried out on 21 selected metabolites at different time points, comparing intra- and inter-individual differences; based on previous findings, the most significantly different metabolites (HCs vs. pSS), such as choline, taurine, alanine, and glycine, were chosen for in-depth analysis. At practically any time, all the factors could discriminate between the two groups.

Li et al. (2022) used ultra-pure liquid chromatography-mass spectrometry UPLC-HRMS on saliva of 32 patients with pSS and 38 healthy adults with age- and sex-matched. They identify 38 metabolites, predominantly amino acids, with potential diagnostic relevance. Their findings revealed that the phenylalanine, tyrosine, tryptophan, and proline pathways are elevated in pSS patients. There is also a disordered metabolism of aspartic acid involved in pSS and several important protein degradation pathways in pSS were upregulated.

Alt-Holland et al. (2023) used 1H-NMR spectroscopy to assess the components of metabolites in unstimulated saliva samples from 30 pSS people and 30 non-Sjogren's disease participants (NS-C). They found out that there is a significant increase in the

levels of glucose, glycerol, taurine, and lactate, as well as significant decreases in the levels of 5-aminopentanoate, acetate, butyrate, and propionate, in subjects with pSS compared to subjects in the NS-C group. It demonstrates that ^{1}H-NMR metabolomics can be used in the clinic to define salivary metabolic signatures associated with disease state and to contribute to differential analysis between people with pSS and those who are not afflicted by this disease.

The latest research on this topic was from Setti et al. (2023) which also used ^{1}H-NMR spectroscopy-based salivary metabolomics on seven cases from a cohort of pSS patients, and six age- and sex-matched HCs from a cohort of volunteers. (Herrala et al., 2021; Mikkonen, 2012) found out that there is a reduction in fucose and amino acids (e.g., glycine and tyrosine) in the saliva of pSS cases. But when compared to the ^{1}H-NMR studies of (Herrala et al., 2021; Mikkonen, 2012) which both reveal a considerable rise of glycine in pSS patients compared to HCs, glycine concentration here appears to have a countertrend. (Setti et al., 2023) demonstrate that butyrate was greater in pSS patients than in HCs, but proline was higher in HCs than in pSS patients, which partially contradicts with (Mikkonen, 2012) findings that butyrate and the amino acid proline were marginally raised in the pSS when compared to HCs.

CHAPTER 3: MATERIALS AND METHOD

3.1 Ethical approval and subjects' selection

The ethics for this study was obtained from the Medical Ethics Committee of Faculty of Dentistry, Universiti Malaya (UM), before the commencement of the research project [DF OS2321/0099 (P)]. Subjects were recruited from the Department of Oral & Maxillofacial Clinical Science, Faculty of Dentistry, UM.

Written informed consent to participate in the study was obtained from all subjects after a thorough explanation of the research project. Subjects were then assessed based on their medical history. Patients with pSS were recruited according to the 2016 (ACR)/ (EULAR) classification. The status of dental caries and periodontal diseases was evaluated using a modified International Caries Detection and Assessment System (ICDAS) and 6-point pocket chart (6PPC), respectively.

The level of dryness of the patient with pSS was measured using the Challacombe scale. Two types of saliva samples which were unstimulated saliva (US), and oral rinse (OR) were collected in two distinct groups of subjects: five HCs and five pSS patients.

3.1.1 The inclusion and exclusion criteria

The inclusion and exclusion criteria of both groups for this study are listed in Table 3.1. All the diseases or use of drugs that could potentially cause dry mouth are excluded.

Table 3.1 Inclusion and exclusion criteria for this study

Criteria	HCs	pSS
Inclusion	18 years and above.	
	The subject is healthy with no history of any type of autoimmune disease (e.g., Sjögren syndrome, rheumatoid arthritis, systemic lupus erythematosus and any intra-oral mucosal diseases.	Diagnosis of pSS is confirmed using the 2016 (ACR)/(EULAR) classification criteria by a rheumatologist and is not secondary to other connective tissue diseases.

Table 3.1 continued

Exclusion	The subject who disagrees and without consent for the study.
	Subjects with smoking habit and with clinically active intraoral infection.
	History of head and neck radiotherapy, HIV or HCV infection, lymphoma, sarcoidosis, graft versus host disease, and use of anticholinergic drugs. Undergoing chemotherapy, use of any investigational drugs, or participation in a clinical trial within 3 months of the study visit.

HCs: Healthy controls; pSS: primary Sjogren syndrome

3.2 Saliva samples collection

Before saliva collection, participants were instructed to refrain from eating, drinking, smoking, and performing oral hygiene for 30 minutes. Saliva collection was done in the morning (9 am-11 am). Two types of samples were collected from each subject: US and OR. For the US, the passive drooling method was performed. Subjects were asked to allow saliva to pool in their mouth for 5 minutes, after which they spit the accumulated saliva into a sterile, Falcon tube until the desired volume of 2-5mL was collected. For the OR sample, subjects swished their mouth vigorously with 10mL of 0.9% (w/v) saline solution (RinsCapR NS) for 3 minutes and then expectorated into a 50mL sterile falcon tube.

After collection, the tubes with US and OR were labelled and maintained on ice in a cooler box to ensure sample integrity before being transported to the High Impact Research Laboratory, UM for sample preparation. The samples were immediately stored at -80°C in an ultra-freezer. The collected US samples and OR samples were divided into two separate bottles each. One was used for LC-Q-TOF MS and the other for ^{1}H-NMR preparation and analysis respectively.

3.3 Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry

3.3.1 Metabolites extraction

The salivary samples for LC-QTOF-MS were prepared according to (Wei et al., 2011). The fresh US sample was centrifuged at 4000rpm for 1 hour at 4°C. The supernatant was stored at -80°C for further analysis. The sample was thawed and dissolved at room temperature before sample preparation and analysis. About 1mL of saliva was mixed with 4mL of methanol (ratio 1:4) using sterilized pipette tips. The mixture was vortex at 1800rpm for 5 minutes. It was then kept for 20 minutes at -20°C fridge. The sample was then centrifuged at 10000rpm for 5 minutes at 4°C. After centrifugation, the supernatant was collected and dried at 4°C using the Labconco Refrigerated Centrivap concentrator (Kansas City, MO, USA) and stored at -80°C in an ultra-freezer pending LC-Q-ToF-MS analysis. The same was carried out for all the OR samples.

3.3.2 Analysis of metabolites

Briefly, the dried samples were redissolved in 50µL of acetonitrile and water. lcA 1200 Infinity Quaternary Liquid Chromatography system with a 6520 Quadrupole Time-of-Flight mass spectrometer connected to a Dual Agilent Jet Stream Electrospray Ionization (Dual AJS ESI) ionization source (Agilent Technologies, CA, USA) was used to analyze the samples mass spectroscopically. After being separated at 40°C to 45°C using an Agilent Zorbax Eclipsed plus C18 Rapid Resolution High Throughput separation column (2.1 x 100mm 1.8µm), the samples were examined for positive modes. The mobile phases were acetonitrile with 0.1% formic acid (B) and water with 1% formic acid (A). A linear gradient from 95% to 5% was set to run for 36 23 minutes, with 5% A held for 5 7 minutes and flushed for 7 minutes at 0.5mL/minute. In the event of the run, the injection volume was set to 2µL, and three injections were performed in complete loop injection mode for each sample. For instrumental control and data collection, Agilent MassHunter Workstation Data Acquisition software (Agilent technologies, CA, USA) was used. The

nebulizer gas pressure (nitrogen) was set to 160 kPa, the ion source temperature was set to 200°C, the dry gas flow was set at 7mL/minutes at source temperature, and the spectral rate was set to 3Hz for MS1 and 10 Hz for MS2. Voltage 3.0kV, gas temperature 300°C, drying gas 8mL/minutes, nebulizer 35 psig, VCap 3500 V, fragmentor 175V, and skimmer 65 V were the electrospray ionization (ESI) conditions. Sample blanks were run after each sample to clean the column and prevent previous samples from being carried over to ensure high-quality data in metabolite profiling.

3.3.3 Data processing and statistical analysis

The MassHunter Qualitative Analysis (MQA) program (version R.06.00, Agilent Technologies, CA, USA) was used to deconvolute raw data into distinct chemical peaks for Molecular Feature Extraction to identify and quantify metabolites. Features were detected within a preset mass accuracy of ±5 ppm and a minimum absolute abundance of 1000 counts. The resulting data were then imported into Agilent Mass Profiler Professional (MPP, version B.12.61) for feature binning, alignment, and consensus generation. MPP was subsequently used for statistical analysis, annotation, and comparison of metabolites across different groups. Feature abundances were normalized to the median abundance across all samples using MPP's baselining option. Unsupervised principal component analysis (PCA) with median centering and scaling was performed to reveal intrinsic variations between groups. Metabolites that showed significant differences between groups were identified using a one-way ANOVA with a 1% Benjamini and Hochberg False Discovery Rate (FDR) adjustment and a fold change (FC) cutoff of 2, with a p-value of less than 0.05. Further, a t-test was used to determine the significance and fold change of metabolites between the two groups. Metabolite identification was carried out using MPP's ID Browser function by matching features based on accurate mass, isotope ratios, abundances, and spacing to entries in the Agilent

METLIN Accurate Mass-Personal Metabolite Database and Library (AM-PCDL, version 5.0).

3.4 Nuclear magnetic resonance

3.4.1 Phosphate buffer preparation for NMR analysis

About 800ml of distilled water was prepared in a bottle. 28.85g disodium hydrogen phosphate (Na2HPO4), 5.25g sodium dihydrogen phosphate (NaH2PO4), 0.172g sodium-3- trimethylsilyl-propionate-2,2,3,3-d4 (TSP), 0.195g sodium azide and 200ml D20 were added into the bottle and made up to 1L using distilled water. TSP concentration was 1mM and sodium azide concentration was 3mM. To ensure all salts will be completely dissolved, the prepared mixture was sonicated for 5 minutes at 40°C. This final solution was adjusted to pH 7.4 using hydrochloric acid or sodium hydroxide. This prepared buffer was secured properly, shielded with aluminium foil to protect from excessive light, and stored in the refrigerator at 4°C.

3.4.2 Metabolites extraction

The US samples for ^{1}H-NMR were prepared according to (Gardner et al., 2020). 1mL of the saliva sample was centrifuged at 13500rpm for 5 minutes to remove insoluble materials, food remnants, and cell debris. Subsequently, 630µL of the US sample was mixed with 70µL of 0.5 mM TSP in 99.9% D2O (5 mg TSP in 1mL D2O; providing a field frequency lock, internal standard, and chemical shift reference at $\delta = 0.00$ ppm) until a total volume of 700µL solution was obtained. The mixture was then vortex at 1800ppm for 2 minutes before being transferred to an NMR tube (Duran® Economic, 178x4.95mm with O.D. 4.95±0.05 and I.D. 4.20±0.05) using a glass Pasteur pipette prior to ^{1}H-NMR analysis. The same was carried out for all the OR samples.

3.4.3 Acquisition and processing

The NMR analysis was conducted using a Bruker AscendTM spectrometer operating at 600MHz, coupled with a sample case autosampler for automated sample handling. The proton NMR (^{1}H-NMR) analysis employed standard parameters optimized for spectral clarity and data quality. This includes using a pulse sequence such as zg or presat, with a spectral width typically set to 12 ppm. The size of the free induction decay (FID) was set to 65536 data points (TD), and 64 scans (NS) were acquired to ensure a sufficient signal-to-noise ratio. The pulse length (P1) was set to 15μs, and the power level (PLW) was maintained at 15 watts. The sample temperature was controlled and maintained at 310.2K throughout the experiment. The duration of each sample analysis was approximately 1-2 minutes. These parameters were designed to facilitate accurate and reproducible characterization of the sample's molecular structure and composition using NMR spectroscopy.

3.4.4 Data pre-processing, statistical analysis and metabolites identification

Among the pre-processing methods for the data are reference peak alignment with TopSpin 4.2, normalization and bucketing with MestReNova 15.0.1, and auto Pareto scaling for both US and OR samples using Soft Independent Modelling of Class Analogy 18 (SIMCA 18) software. In addition, prior to bucketing, spectral noise, TSP regions (-0.05 to 0.05 ppm), and water peak regions (4.6 to 4.9 ppm) in the spectra were all eliminated. Additionally, the intrinsic metabolic variation in the ^{1}H-NMR spectra data was observed using SIMCA 18 software to generate principal components analysis (PCA), orthogonal partial least squares-discriminant analysis (OPLS-DA), Permutation table, and variable importance in projection (VIP) score.

To maximize the variation between groups and identify the key metabolites contributing to the variation, OPLS-DA plots were employed. Every noteworthy bin area

that has more than one VIP score was looked up in the Human Metabolome Database (HMDB) and the Biological Magnetic Resonance Data Bank (BMRB) online databases. To verify that metabolites were present in the biological samples, the closest peak range compounds were found by overlaying spectra with TopSpin 4.2. Finally, the MetaboAnalyst 5.0 website would be used to search for the involved route for these important metabolites.

CHAPTER 4: RESULTS

The distribution of subjects in both groups with respect to age, gender and (ACR)/(EULAR) classification are described in Table 4.1. The age range for all samples was between 18-62 years old, with a mean age of 44.8 years. Each participant in the pSS group will have corresponding HCs matched by gender, age, and race. All five subjects diagnosed with pSS tested positive for the Anti-SSA (Ro) antibody, with one showing strong positivity. Four out of five subjects were positive for the Schirmer test $\leqslant$ 5 mm/5 minute and three of them had unstimulated whole saliva less than 0.1ml per minute. All five of them fulfilled the criteria of having at least 4 scores from the 2016 (ACR)/ (EULAR) classification criteria to be diagnosed as pSS.

Table 4.1 Demographic and (ACR)/(EULAR) classification of all the subjects

Patient ID	Age	Gender	Race	ACR-EULAR Classification						Challacombe scale
				Anti SSA (Ro) Antibody Positivity (3 SCORE)	Focal lymphocytic sialadenitis with a focus score ≥ 1 foci/mm^2 (3 SCORE)	Ocular staining score ≥ 5 (or van Bijsterveld score ≥ 4 (1 SCORE)	Schirmer test ≤ 5 mm/5 min (1 SCORE)	Unstimulated salivary flow rate ≤ 0.1 mL/min (1 SCORE)	TOTAL SCORE =>4	
HC01	22	Female	Chinese	NA	NA	NA	NA	NA	NA	NA
HC03	62	Female	Malay	NA	NA	NA	NA	NA	NA	NA
HC05	59	Female	Chinese	NA	NA	NA	NA	NA	NA	NA
HC06	31	Female	Chinese	NA	NA	NA	NA	NA	NA	NA
HC07	55	Female	Malay	NA	NA	NA	NA	NA	NA	NA
pSS01	18	Female	Chinese	Positive	/	NA	NA	NA	6	3
pSS02	64	Female	Malay	Positive	NA	NA	/	/	5	5
pSS03	53	Female	Chinese	Positive	NA	NA	/	/	5	4
pSS04	31	Female	Chinese	Positive	NA	NA	/	/	5	1
pSS05	53	Female	Malay	Strong positive	NA	NA	/	NA	4	3

HC: Healthy control, pSS: primary Sjogren syndrome, NA: Not applicable

4.1 Liquid Chromatography Quadrupole Liquid time-of-flight- mass spectrometry

Figures 4.1- 4.4 illustrate chromatograms of US of HCs and pSS and OR of HCs and pSS.

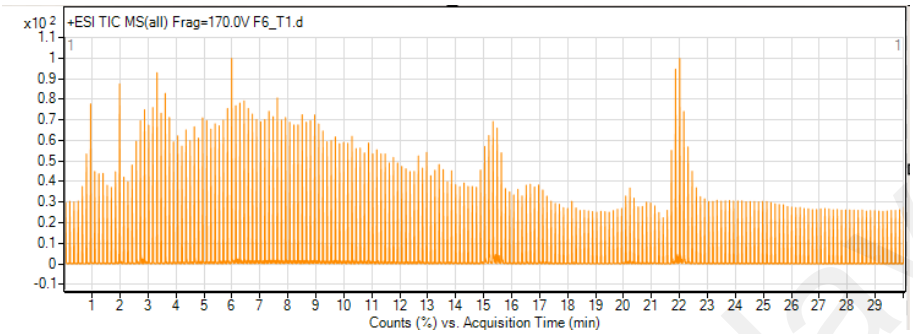


Figure 4.1: Chromatogram of raw data for unstimulated saliva in healthy control

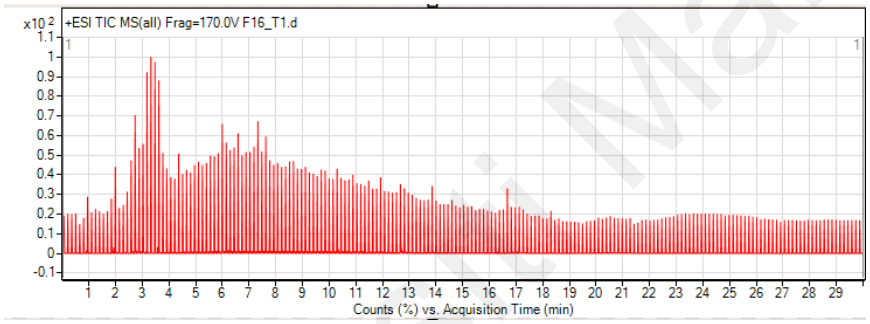


Figure 4.2: Chromatogram of raw data of oral rinse in healthy control

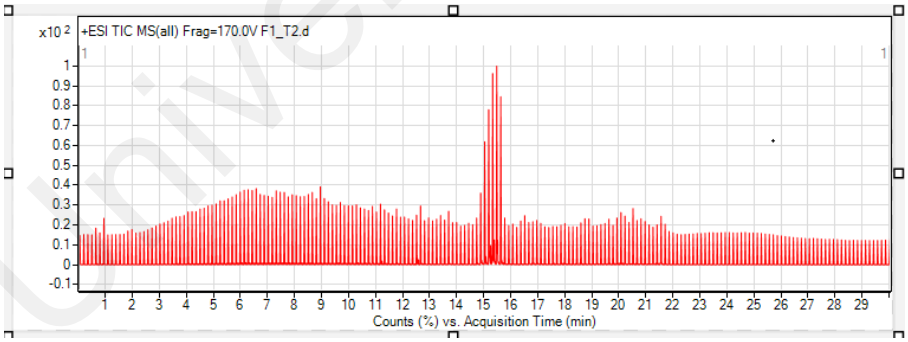


Figure 4.3: Chromatogram of raw data for unstimulated saliva of primary Sjogren syndrome

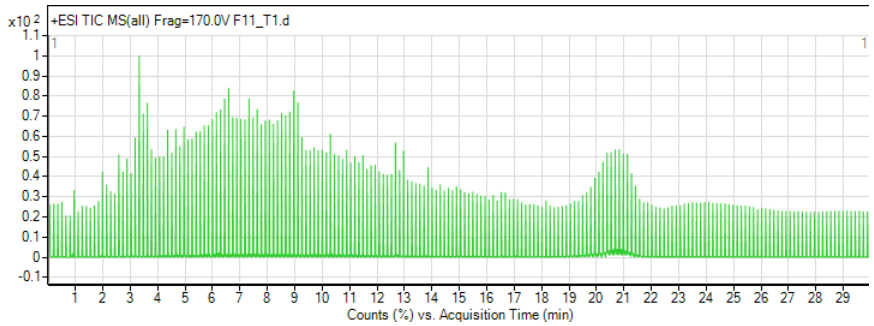


Figure 4.4: Chromatogram of raw data for oral rinse of primary Sjogren syndrome

Then, descriptive statistical analysis was carried out for both the groups in both types of samples (n= 20). A total of 269 metabolites were obtained (Appendix C). Among the 269 metabolites identified, 68 compounds match with Metlin AMRT PCDL database and 175 metabolites were given as chemical formula (Appendix C).

Table 4.2: Upregulations and downregulation of metabolites in the two group in both types of samples.

Groups		Number of metabolites	
		Upregulated	Downregulated
US HCs	OR HCs	96	137
US pSS	OR pSS	64	205
US HCs	US pSS	64	205
OR HCs	OR pSS	71	198

HCs: healthy controls; pSS: primary Sjogren syndrome; US: Unstimulated Saliva; OR: Oral Rinse

Upregulation and downregulation of metabolites in the two groups in both types of samples is given in table 4.2. The upregulation ranged from 64 to 96 metabolites while the downregulation ranged from 137 to 205 metabolites.

Next, unsupervised PCA was used for quality control of samples. PCA is most frequently used to compress, with the least amount of information loss, the information present in many original variables into a smaller set of new composite dimensions. PCA was performed to explore the tendency of metabolic profiling variations between all the groups; US of HCs, US of pSS, OR of HCs, OR of pSS.

4.1.1 Unstimulated saliva of healthy control versus oral rinse of healthy control

Descriptive statistical analysis was carried out between US of HC against OR of HC in 5 samples each. A total of 28 metabolites were obtained, in which 20 metabolites were upregulated and 8 were downregulated (Appendix D). Among the 28 metabolites identified, 6 compounds matched with Metlin AMRT PCDL database and 17 metabolites were given as chemical formula (Appendix D). The PCA plot (Figure 4.5) shows overlapping clusters.

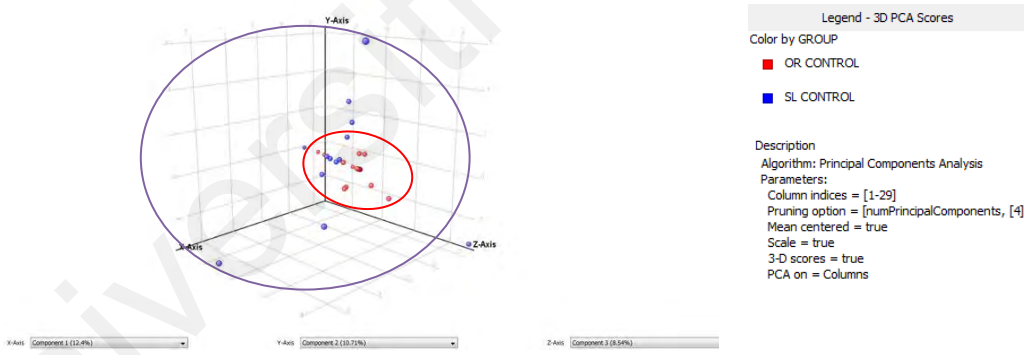


Figure 4.5: PCA in three dimensions of unstimulated saliva of healthy control (blue) versus oral rinse of healthy control (red) created using MPP (n=10)

4.1.2 Unstimulated saliva of primary Sjogren syndrome versus oral rinse of primary Sjogren syndrome

Descriptive statistical analysis was carried out between US of pSS versus OR of pSS in 5 samples each. A total of 57 metabolites were obtained of which 29 metabolites were upregulated and 28 were downregulated. (Appendix E). Among the 57 metabolites identified, 12 compounds matched with Metlin AMRT PCDL database, and 37 metabolites were given

as chemical formula (Appendix E). The PCA plot (Figure 4.6) demonstrates overlapping clusters among the groups, indicating that many of the compounds share similar characteristics; however, some major differences can be seen in the distinct positioning of certain data points, suggesting the presence of unique features within those specific compounds.

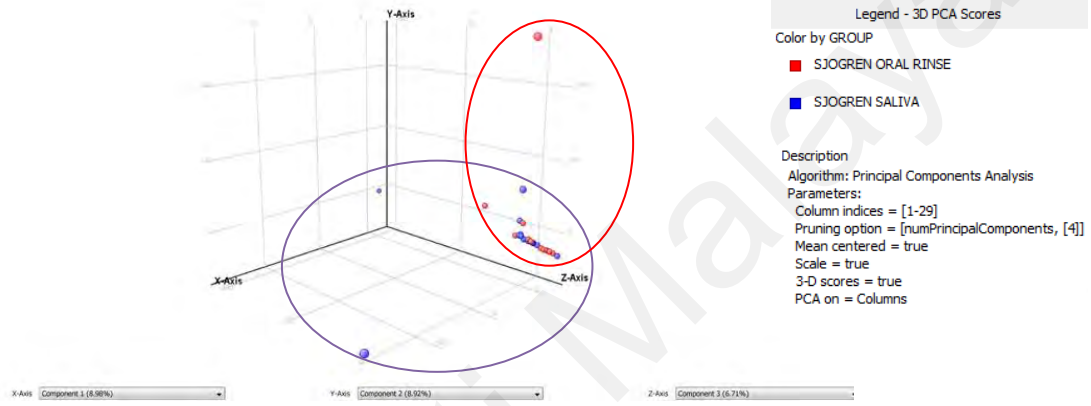


Figure 4.6: PCA in three dimensions of unstimulated saliva of primary Sjogren syndrome (blue) versus oral rinse of primary Sjogren syndrome (red) created using MPP (n=10)

4.1.3 Unstimulated saliva of healthy controls versus unstimulated of saliva of primary Sjogren syndrome

Descriptive statistical analysis was carried out between US of HC versus US of pSS in 5 samples in each group. A total of 29 metabolites were obtained of which 11 metabolites were upregulated and 18 were downregulated. (Appendix F). Among the 29 metabolites identified, 9 compounds matched with Metlin AMRT PCDL database and 18 metabolites were given as chemical formula (Appendix F). The PCA (Figures 4.7) plot shows overlapping clusters within a small area, indicating that the groups share many similarities, but there are also

major differences evident in the distinct positioning of certain outliers, highlighting unique features in those specific data point.

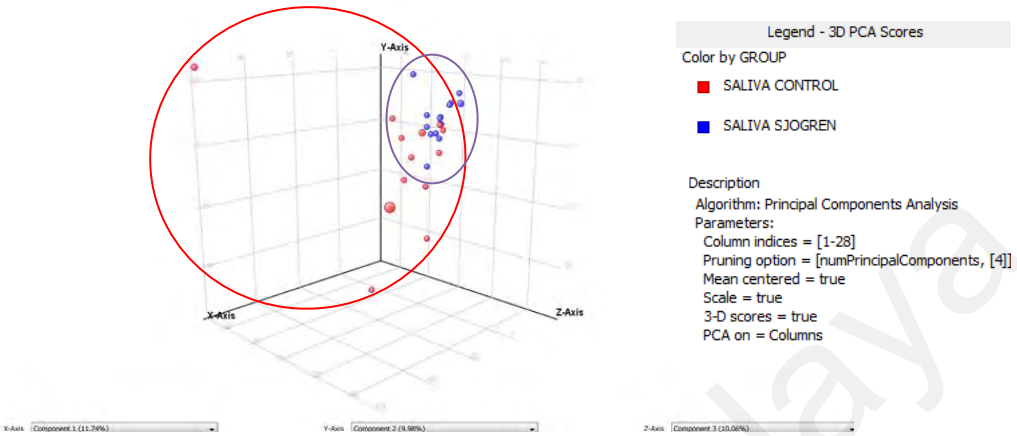


Figure 4.7: PCA in three dimensions of unstimulated saliva of healthy controls (red) versus unstimulated saliva of primary Sjogren syndrome (blue) created using MPP (n=10)

4.1.4 Oral rinse of healthy controls versus oral rinse of primary Sjogren Syndrome

Descriptive statistical analysis was carried out between OR of HC versus OR of pSS in 5 samples per group. A total of 53 metabolites obtained of which 28 metabolites were upregulated and 25 were downregulated (Appendix G). Among the 53 metabolites identified, 10 compounds match with Metlin AMRT PCDL database and 35 metabolites were given as chemical formula (Appendix G).

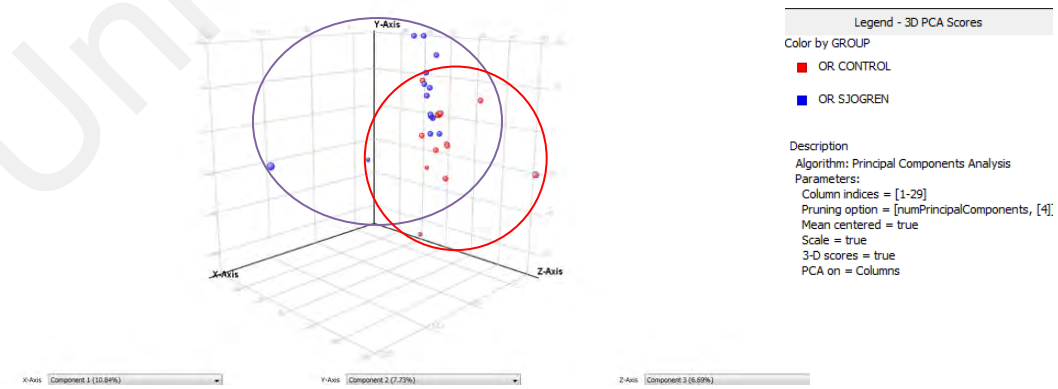


Figure 4.8: PCA in three dimensions of oral rinse of healthy controls (red) versus oral rinse of primary Sjogren syndrome (blue) created using MPP (n=10)

The PCA plot (Figures 4.8) shows overlapping clusters within a major area, indicating that the groups have many similarities; however, there are also significant differences, as evidenced by distinct data points that stand out, highlighting unique features within those specific observations.

When comparing US and OR, the OR samples contained almost the same metabolites as the US samples. Meanwhile, some metabolites were detected in only one type of samples and were not detected in the other. The top five metabolites of US of HCs, US of pSS, OR of HCs, OR of pSS groups were determined and are listed in Table 4.3.

Table 4.3: List of top 5 metabolites using LC-QTOF-MS for both group and both types of samples

No	Saliva		Oral Rinse	
	HC	**pSS**	**HC**	**pSS**
1	8-Epiiridotrial glucoside	Purine	Quassin	7,4'-Dihydroxyflavone
2	Levoamine (Chloramphenicol D base)	Diethyl Maleate C8 H12 O4	N4-Phosphoagmatine	Diethyl Maleate C8 H12 O4
3	N4-Phosphoagmatine	Epicatechin 3-O-beta-D-glucopyranoside	2-[3-Carboxy-3-(methylammonio)propyl]-L-histidine	Quassin
4	Tubaic Acid	Dehydroneotenone	Dehydroneotenone	Orthothymotinic Acid
5	5-L-Glutamyl-taurine	N[(Methylnitrosoamino)carbonyl]-D-glucosamine)	MID1766:5-ethyl-5-(pentan-2-yl)-1-((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-	Dehydroneotenone

HC: Healthy control; pSS: primary Sjogren syndrome

When using the US samples, the top 3 metabolites found in pSS are purine, diethyl maleate and epicatechin 3-O-beta-D-glucopyranoside, while 8-epiiridotrial glucoside, levoamine (chloramphenicol D base) and N4-phosphoagmatine are the top 3 metabolites found in HCs. Interestingly, we also found diethyl maleate in pSS when using OR samples, besides 7,4'-dihydroxyflavone and quassin.

Using one-way ANOVA, comparing among groups; US of HCs versus pSS and OR of HCs versus pSS, only one metabolite was found to be statistically significant which is purine (p-value = 0.010), and fold count of 618.6047. Purine was found to be upregulated in pSS group when compared to HCs when using US as the samples. When using OR samples between HCs and pSS, the same purine was found to be upregulated in pSS however it was not significant (p-value= 0.310). Purine is also found in HCs using US and OR, but it was not listed as it is not the top 5 metabolites found.

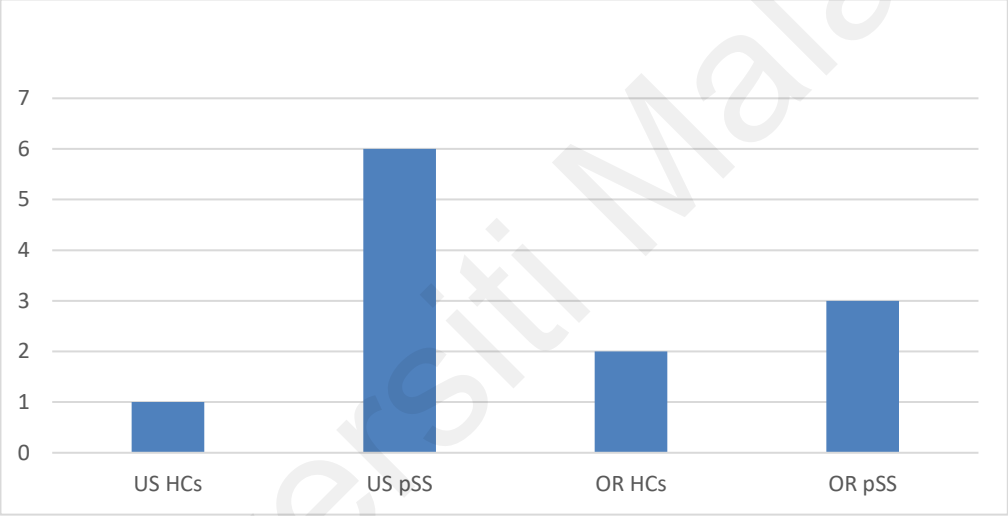


Figure 4.9: Levels of purine in both groups and both sample types.

4.2 ^{1}H-Nuclear Magnetic Resonance

The NMR metabolomics analysis provided insightful data regarding the metabolic profiles of this study. Analysis focused on the comparison between the US of HC against US of pSS, utilizing advanced statistical methods to evaluate the specificity and sensitivity of the proposed model. However, PCA was performed to explore the tendency of metabolic profiling variations between all the groups; US of HCs, US of pSS, OR of HCs, OR of pSS.

4.2.1 Unstimulated saliva of healthy controls versus oral rinse of healthy controls

When the PCA plot was done comparing between US of HCs and OR of HCs, again it showed overlapping clusters, which was beneficial as it indicates the necessary similarities between the two groups, which is essential for our analysis.

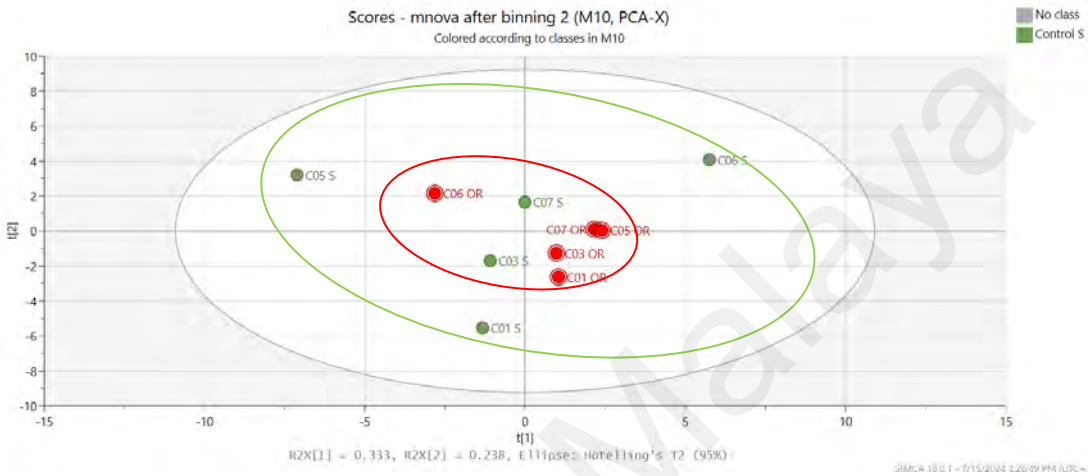


Figure 4.10: PCA in three dimensions of unstimulated saliva of healthy controls (green) versus oral rinse of healthy controls (red) using MPP (n=10)

4.2.2 Unstimulated saliva of primary Sjogren syndrome versus oral rinse of primary Sjogren syndrome

The PCA plot (Figure 4.11) demonstrates overlapping clusters among the groups, indicating that many of the compounds share similar characteristics; however, some major differences can be seen in the distinct positioning of certain data points, suggesting the presence of unique features within those specific compounds. This data is similar to LC-QTOF-MS data.

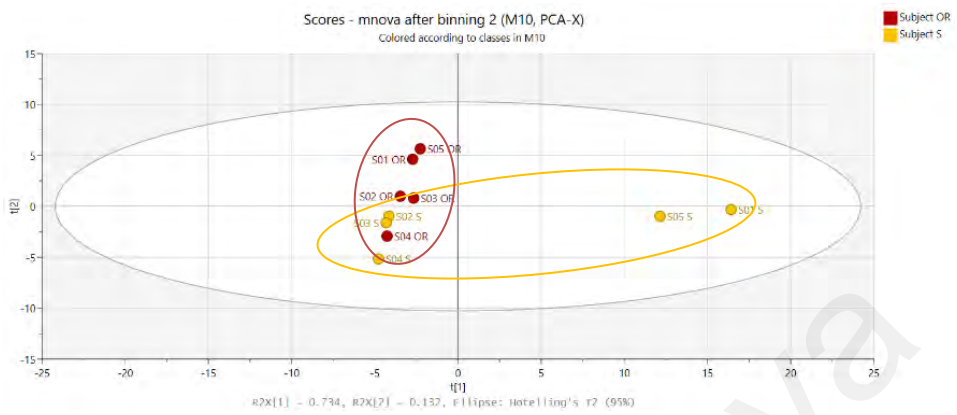


Figure 4.11: PCA in three dimensions of unstimulated saliva of primary Sjogren syndrome (Yellow) versus oral rinse of primary Sjogren syndrome (Red) created using MPP (n=10)

4.2.3 Oral rinse of healthy controls versus oral rinse of primary Sjogren Syndrome

The PCA plot (Figure 4.12) shows overlapping clusters within a major area, indicating that the groups have many similarities; however, there are also significant differences, as evidenced by distinct data points that stand out, highlighting unique features within those specific observations. We found similar results when using LC-QTOF-MS.

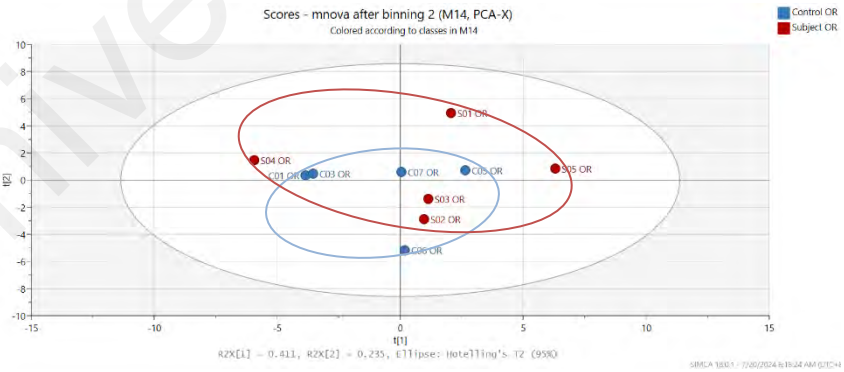


Figure 4.12: PCA plot in three dimensions for oral rinse of control (blue) versus oral rinse of primary Sjogren syndrome (red) created using MPP (n=10)

4.2.4 Unstimulated saliva of healthy controls versus unstimulated saliva of primary Sjogren syndrome

The PCA plot (Figure 4.13) shows distinct, non-overlapping clusters, indicating clear differences between the groups. Each cluster represents unique features within those specific observations.

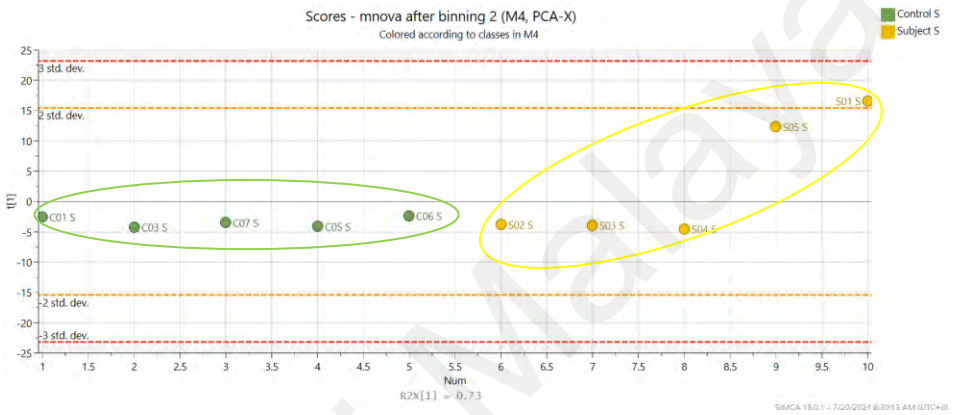


Figure 4.13: PCA plot of unstimulated saliva of healthy control (green) versus unstimulated saliva of primary Sjogren syndrome (yellow) created using MPP (n=10)

Upon observing the distinct difference, we then proceed with further analysis using an OPLS-DA plot (Figure 4.14) generated from the NMR spectral data which again, demonstrates a clear differentiation between the groups, with distinct clusters representing each class, and only minor overlapping, indicating that the model effectively captures the variations between the groups while maintaining high classification accuracy. This clear separation indicates the presence of significant metabolic differences between the groups.

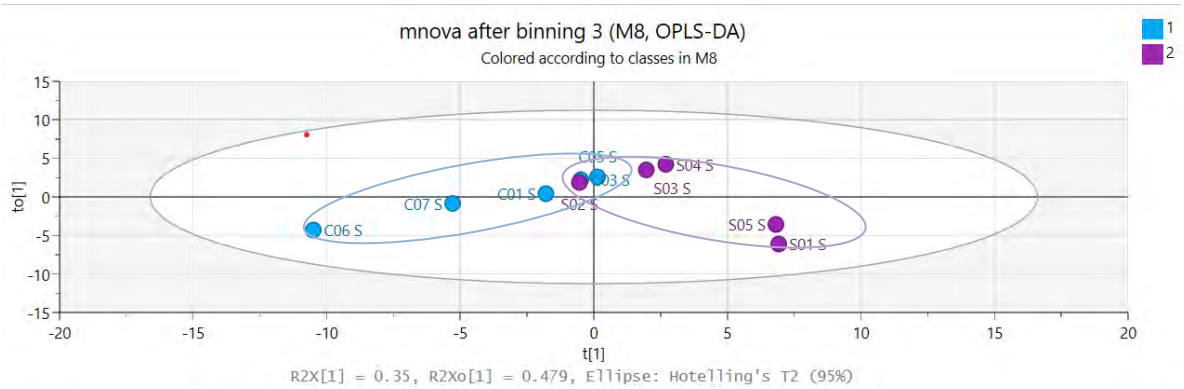


Figure 4.14: OPLS-DA plot shows discriminating between unstimulated saliva of healthy controls (blue) and unstimulated saliva of primary Sjogren syndrome (purple) created using MPP (n=10)

We further analysed the difference between the two groups using Variable Importance in Projection (VIP) plot. The VIP plot (Figure 4.15) generated from the OPLS-DA analysis highlights the importance of each variable in the model. Variables with VIP scores greater than 1 are considered significant contributors to the model, indicating their strong influence on differentiating between the groups. The plot shows that several variables have VIP scores well above 1, suggesting they are crucial in distinguishing between the HCs and pSS groups.

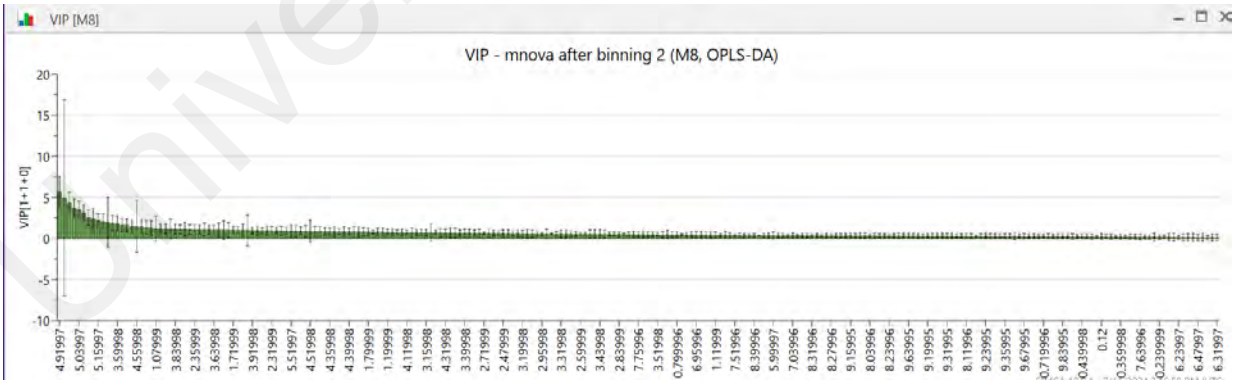


Figure 4.15: VIP Plot for unstimulated of healthy controls versus unstimulated of saliva of primary Sjogren syndrome.

From the VIP plot, a total of 241 bins were identified, among which 63 bins had a VIP score greater than 1 (Appendix H). Bins with VIP scores greater than 1 are considered important variables in the OPLS-DA model. These bins are likely to contain metabolite

signals that are key differentiators between the groups. These bins were then matched with each sample's spectral to find the ppm and exclude the noise. A thorough data search was done using HMDB and BMRB databank to find the associate possible metabolites. The top 5 metabolites found are listed in Table 4.4.

Table 4.4: Metabolites found in unstimulated saliva of healthy controls and primary Sjogren syndrome

No	Healthy controls (HCs)			Primary Sjogren syndrome (pSS)		
	Primary ID	Metabolites	ppm	Primary ID	Metabolites	ppm
1	1.9198	N-Acetyl-L-Glutamine	1.92	1.35999	Alpha-Hydroxyisobutyric acid	1.31,1.36
2	3.55998	Glycine	3.55	3.55998	Glycine	3.55
3	3.79998	Phosphoguanidinoacetate	3.78	1.0725	Hydrogen selenide	1.075
4	2.39999	(Alpha-D-mannosyl) 7-beta-D-mannosyl-diacetylchitobiosyl-L-asparagine, isoform A (protein)	3.72, 3.71, 3.69, 3.68, 1.48,1.46	2.39999	(Alpha-D-mannosyl) 7-beta-D-mannosyl-diacetylchitobiosyl-L-asparagine, isoform A (protein)	3.72, 3.71, 3.69, 3.68, 1.48,1.46
5	2.4779	Succinic acid	2.39	2.4779	Succinic acid	2.39

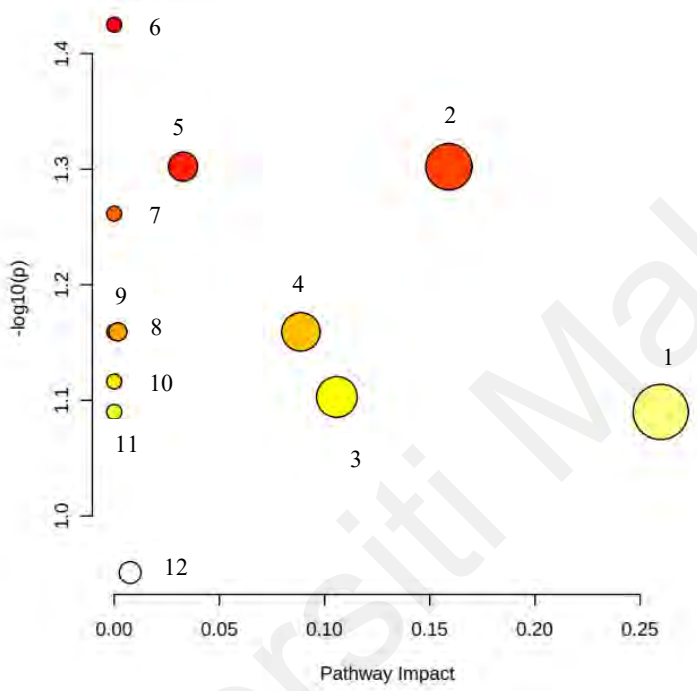
The identified metabolites were mapped to metabolic pathways in MetaboAnalyst database. Notably, metabolites that were upregulated in the US of pSS versus US of HCs were mapped to 12 metabolic pathways.

Table 4.5: The metabolic pathways analyzed with MetaboAnalyst 5.0 among upregulated metabolites.

Pathway	US of HCs versus US of pSS		
	Match	p-value	Impact
Butanoate metabolism	1/15	0.03759	0.0
Citrate cycle (TCA cycle)	1/20	0.049881	0.03273
Selenocompound metabolism	1/20	0.049881	0.15909
Propanoate metabolism	1/22	0.054764	0.0
Alanine, aspartate and glutamate metabolism	1/28	0.069301	0.0
Lipoic acid metabolism	1/28	0.069301	0.0017
Glutathione metabolism	1/28	0.069301	0.08873
Porphyrin metabolism	1/31	0.076507	0.0
Glyoxylate and dicarboxylate metabolism	1/32	0.078899	0.10582

Table 4.5 continued

Cysteine and methionine metabolism	1/33	0.081287	0.0
Glycine, serine and threonine metabolism	1/33	0.081287	0.25981
Primary bile acid biosynthesis	1/46	0.11191	0.00758



1. Glycine, serine and threonine metabolism.
2. Selenocompound metabolism
3. Glyoxylate and dicarboxylate metabolism
4. Glutathione metabolism.
5. Citrate cycle (TCA cycle)
6. Butanoate metabolism.
7. Propanoate metabolism.
8. Alanine, aspartate and glutamate metabolism.
9. Lipoic acid metabolism.
10. Porphyrin metabolism.
11. Cysteine and methionine metabolism.
12. Primary bile acid biosynthesis

Figure 4.16: Overview of the pathway analysis which is associated with of unstimulated saliva of primary Sjogren syndrome using MetaboAnalyst 5.0.

The figure displays pathways that correspond to annotated metabolites and are organized according to the p-value (y-axis), which represents pathway enrichment analysis, and the pathway impact values (x-axis), which represents pathway topology analysis. The metabolic pathways are shown as bubbles based on their scores from enrichment as seen in the y axis, and topology (pathway impact) values as seen in x axis. The p-value determines the node colour of each pathway (red = lowest p-value and highest statistical significance) and indicates that metabolite changes in the relevant pathway are more significant. The size of

the bubble correlates to the pathway impact score (biggest indicating the highest impact) and is related to the centrality of the metabolites involved. Specify pathway analysis parameters include Relative-betweenness Centrality for topology analysis and Hypergeometric Test for enrichment.

In butanoate metabolism there is one significant metabolite out of 15. The p-value of 0.037 indicates statistical significance, but the impact score of 0.00 suggests that the position or connectivity of this metabolite within the pathway may not be central. In citrate cycle (TCA Cycle) there is one significant metabolite out of 20, with a marginally significant p-value of 0.049. The impact score is low, indicating a limited influence on the overall pathway. While in Selenocompound Metabolism there is one significant metabolite out of 20, with a significant p-value of 0.049. The impact score of 0.15 is higher compared to the previous pathways, suggesting a more influential role of the metabolite within this pathway. In propanoate metabolism there is one significant metabolite out of 22, with a p-value slightly above the conventional threshold of 0.05. The impact score is 0.00, indicating minimal influence.

Other than that, in alanine, aspartate, and glutamate metabolism we have one significant metabolite out of 28, with a non-significant p-value and an impact score of 0.0. Meanwhile, in lipoic acid metabolism there is one significant metabolite out of 28, non-significant p-value of 0.069301, and a very low impact score of 0.0017. While in glutathione metabolism, one significant metabolite out of 28, non-significant p-value of 0.069, but a relatively higher impact score of 0.08 indicating some influence within the pathway. In porphyrin metabolism there is one significant metabolite out of 31, non-significant p-value 0.076507, and no impact within the pathway. In glyoxylate and dicarboxylate metabolism one significant metabolite

out of 32, had a non-significant p-value of 0.078, but a relatively higher impact score of 0.10. The cysteine and methionine metabolism pathway, with one significant metabolite out of 33 (p-value 0.081287, impact score 0.0), shows no statistical significance or impact. On the other hand, the glycine, serine, and threonine metabolism pathway also have one significant metabolite out of 33 (p-value 0.081287), but a higher impact score of 0.25981 suggests it might have some biological relevance despite the non-significant p-value. Lastly, the primary bile acid biosynthesis pathway, with one significant metabolite out of 46 (p-value 0.11191, impact score 0.00758), has neither statistical significance nor a notable impact. Lastly, in glyoxylate and dicarboxylate metabolism one significant metabolite out of 32, had a non-significant p-value of 0.078, but a relatively higher impact score of 0.10.

Using the US samples, the top 5 metabolites found in pSS are alpha-hydroxyisobutyric acid, glycine, hydrogen selenide, (alpha-D-mannosyl)7-beta-D-mannosyl-diacetylchitobiosyl-L-asparagine, isoform A (protein), and succinic acid. In HCs, the top 5 metabolites are n-acetyl-l-glutamine, glycine, phosphoguanidinoacetate, (alpha-D-mannosyl)7-beta-D-mannosyl-diacetylchitobiosyl-L-asparagine, isoform A (protein), and succinic acid. Interestingly, n-acetyl-l-glutamine and phosphoguanidinoacetate were found exclusively in the saliva of the HCs group.

CHAPTER 5: DISCUSSION

In this study, two types of samples were collected (US and OR) from two different groups (HCs and patients with pSS). Metabolic profiling of these two types of samples in the two groups was carried out using LC-QTOF-MS and ^{1}H-NMR. Further analysis was carried out to find the differences in metabolites between the HCs and pSS groups. Statistical analysis was carried out to obtain the statistically significant difference of the metabolite between the two groups.

5.1 Unstimulated saliva versus oral rinse

Numerous investigations found variations in the salivary metabolic profiles of stimulated and US samples using NMR, MS, or combined analytical techniques. Maruyama et al. (2022) used salivary metabolomics based on MS to examine the metabolic profiles of mouth-rinsed water, stimulated saliva, and unstimulated saliva from healthy individuals. Using these sampling techniques, 153 common metabolites were examined; nevertheless, certain metabolites were found in just one or two of them and not in the others. Figueira et al. (2017) presented the results of a comparative metabolomics study utilizing LC-MS and targeted NMR spectroscopy on saliva from parotid, stimulated, and unstimulated glands. The three procedures used to collect saliva samples differed in the metabolite composition ratios, despite the almost similar compounds that were found. Takeda et al. (2009) demonstrated increased amounts of nearly all metabolites in saliva samples from a cohort of healthy males when compared between unstimulated and stimulated, and male saliva samples had higher quantities of several metabolites than female saliva samples. Therefore, further comparisons between the metabolic profiles of unstimulated and stimulated saliva samples from pSS

patients and those who are not affected by pSS may reveal complementary metabolite information and should be considered in future studies.

In LC-QTOF-MS, when comparing US to OR for metabolite analysis, both samples have shown promise in reflecting individual differences and containing similar metabolites. The PCA plot analysis for OR of HCs versus US of HCs shows overlapping of the compound which is beneficial as it indicates the similarities among compounds between the two groups.

Moreover, in ^{1}H-NMR, the PCA plot analysis for OR of HCs versus US of HCs, again reveals an overlap of compounds, highlighting the similarities between the two groups. This coincides with other studies (Jo et al., 2019; Maruyama et al., 2022) in which mouth rinse contained the same metabolome as that of US and stimulated saliva samples. Some metabolites are not detected in the OR samples, possibly due to the dilution of low-concentration metabolites in the oral cavity, which prevented their detection in OR. Some characteristic metabolites which only found in OR are probably due to the rinsing action. This shows that OR may be an appropriate alternative to saliva in oral metabolome profile analysis in pSS patients with severe dry mouth resulting is extremely less quantity of saliva collection.

5.2 Metabolic profiling in healthy controls

In LC-QTOF-MS, N4-phosphoagmatine was found in both OR and US of HCs. Phosphoagmatine is functionally related to agmatine. Agmatine has shown significant anti-inflammatory properties across various studies. Research indicates that agmatine attenuates allergic inflammation in the airways by reducing cytokine release, nitric oxide expression, and oxidative stress (Elmahdy et al., 2022). Moreover, in cholestasis-induced lung injury, agmatine ameliorated inflammatory indicators, oxidative stress biomarkers, and tissue

fibrosis, highlighting its protective role (Ommati et al., 2023). In a study on microglial cells, agmatine was found to suppress oxidative stress and inflammatory responses, pushing microglia toward an anti-inflammatory phenotype (Milosevic et al., 2022).

This study also found gamma-L-glutamyl-taurine, also known as litoralon, found exclusively in US of HCs. Gamma-L-glutamyl-taurine has been studied for its effects on inflammation. Taurine, a key component of litoralon, plays a crucial role in inflammation by reacting with hypochlorous acid to form taurine chloramine (TauCl), which exhibits anti-inflammatory properties (Springer-Verlag, 2014). Additionally, taurine has been shown to contribute to the clinical efficacy of prodrugs in treating colitis by inhibiting NF-kappaB activation, a major proinflammatory signal in the inflamed large intestine (Marcinkiewicz & Kontny, 2014). The presence of taurine in this dipeptide suggests potential anti-inflammatory benefits, making it a promising area for further research in the context of inflammatory diseases.

In ^{1}H-NMR, while categorizing the metabolites, we found N-acetyl-L-glutamine (NAPA) to be exclusive in the US of HCs. The NAPA has shown promising anti-inflammatory effects in various studies. Research indicates that NAPA can act as a disease-modifying drug in osteoarthritis (OA) by inhibiting IKKα kinase, reducing catabolic enzymes, and counteracting key cartilage effector (Pagani et al., 2019). Additionally, L-glutamine has demonstrated anti-inflammatory properties in acute lung injury (ALI) by attenuating inflammatory markers and improving lung tissue pathology through the TLR4/MAPK signalling pathway (Huang et al., 2021).

5.3 Metabolic profiling in primary Sjogren syndrome

Using LC-QTOF-MS, we found diethyl maleate in both sample types (US and OR) of pSS. Diethyl maleate is a maleate ester resulting from the formal condensation of both carboxy groups of maleic acid with ethanol. It has a role as a glutathione-depleting agent. Diethyl maleate is known to react with glutathione, a crucial antioxidant in the body, thereby depleting its levels. This depletion can lead to increased oxidative stress, as glutathione (GSH) is an important antioxidant protecting cells from damage to reactive oxygen forms. It exists in cells in two main forms: reduced GSH and oxidized GSH (GSSG). An increased ratio of GSSG to GSH is usually indicative of oxidative stress (Jiang et al., 2016).

We also found hydrogen selenide, a metabolite of selenium to be upregulated in US of pSS when using ^{1}H-NMR. Hydrogen selenide is a key intermediate in the selenium methylation metabolism of inorganic and organic selenium compounds. Accumulation of the hydrogen selenide resulting from inhibition of the selenium methylation metabolism, detoxification metabolic pathway of selenium, is found in animals following repeated administration of a toxic dose of selenocystine. The excess of the hydrogen selenide produced by inhibition of the selenium methylation metabolism contributes to the hepatotoxicity caused by selenocystine. Other than that, deficiency in selenium and dysregulation of selenoproteins have been linked to problems with cartilage's redox balance. The most prevalent type of arthritis, OA, is a degenerative condition marked by cartilage degradation that results from an imbalance in matrix metabolism. One of the main factors contributing to the start of the OA pathogenesis is oxidative stress (Kang et al., 2020).Further investigation needs to be done to see whether disruption of selenium metabolism that cause increase in oxidative stress can be linked with chronic inflammatory diseases.

5.4 Differences in metabolites of healthy controls and primary Sjogren syndrome and their significance

Using LC-QTOF-MS, we discovered anomalies in purine metabolism which we found to be upregulated in pSS group when compared to the HCs group. Gout is a condition brought on by disruptions in purine metabolism. Because hyperuricemia produces crystals, gout can present as arthritis (Alberich et al., 2017). In pSS peripheral blood mononuclear cells, purinergic receptor P2X ligand-gated ion channel 7 (P2X7R) mRNA and protein levels were shown to be considerably greater than those in the normal group (Yu et al., 2015). The P2X7R has a role in the development of pSS. Through the activation of the NALP3 inflammasome, P2X7 activation is known to result in the processing and secretion of the proinflammatory cytokines IL-1β and IL-18 from monocytes/macrophages, which is believed to be involved in a variety of inflammatory illnesses (Lester et al., 2013). Similar findings were also reported by Li et al., (2022), where they found abnormalities in purine metabolism. Purine is also found to be upregulated in the pSS group when compared with the healthy subject. Upregulation of purine metabolism indicates an inflammatory response in pSS patients.

In ^{1}H-NMR, the OPLS-DA has been utilized in various studies, such as in the evaluation of tissue classification approaches for cancer diagnostics based on molecular profiles Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) is a valuable statistical modelling tool in NMR analysis, aiding in the differentiation between experimental groups based on high-dimensional spectral data (Worley & Powers, 2016). In this study, OPLS-DA plot, when comparing US of HCs versus US of pSS, we found that while there are distinct clusters representing differences between the two groups, some intersecting compounds are observed. This intersection indicates that despite the overall separation, certain metabolites are common to both groups. These shared compounds may play

fundamental roles in basic physiological processes, reflecting the underlying metabolic similarities despite the differences highlighted by the OPLS-DA analysis.

Additionally, the VIP scores highlight the key metabolites contributing to the differentiation between groups, with 63 VIP scores exceeding the threshold of 1, underscoring their significant role in our analysis. When we mapped the upregulated metabolites in the US of pSS versus US of HCs to metabolic pathways in MetaboAnalyst database, we found 12 metabolic pathways.

The most notable pathway is butanoate metabolism, which was statistically significant but showed no impact. Additionally, selenocompound metabolism is statistically significant and has a relatively higher impact score, suggesting potential biological relevance. Moreover, pathways with potential relevance include glutathione metabolism and glyoxylate and dicarboxylate metabolism. Although their p-values are not significant, their impact scores suggest some degree of relevance. However, further research is required.

From this study, we found that there was a significant difference in the salivary metabolites profile (purine) between patients with pSS and HCs thus analyzing purine metabolites in saliva could serve as a non-invasive diagnostic method for discriminating between patients with pSS from HCs and monitor disease progression.

5.5 Limitations and Recommendations

The main limitation of this pilot study was the small sample size, allowing for limited statistical interpretation. A larger population will be required to observe better separation of groups in the PCA analysis. Nevertheless, OPLS-DA can provide good insight.

The excellent sensitivity of MS technology allows for qualitative investigation of the salivary metabolome even with a small sample size. This leads to the technology's widespread use. Because of its high sensitivity, MS can detect many metabolites (between 300 and 1000+ in the case of GC-MS or LC-MS analysis), its inexpensive platform costs make it a great choice for tailored metabolomics

Although NMR spectroscopy offers many benefits, there are certain drawbacks when using it as a screening method. Superconducting magnets used in contemporary NMR spectrometers need to be cooled using liquid helium, which makes them incredibly intricate and costly devices to buy and run. Taking measurements and deciphering the obtained spectra in ^{1}H-NMR required multiple manual software analyses and database which are difficult and very time-consuming tasks. Some of the free software has limited options in analysing the spectral and that limits us from doing metabolites profiling for all groups using ^{1}H-NMR. We couldn't find any statistically significance metabolites using ^{1}H-NMR due to the small sample size. This is because ^{1}H-NMR is less sensitive compared to other techniques like mass spectrometry (MS). ^{1}H-NMR can only detect around 50-200 metabolites. In ^{1}H-NMR, metabolites present in low concentrations may fall below the detection limit. Additionally, a large volume of saliva is usually required for measurement, which might be difficult to obtain from people who have dry mouths.

Future technological advancements, such as (e.g. picoSpin™ miniature NMR spectrosmeter, Thermo Scientific) along with stronger data analysis capabilities, will assist in overcoming current constraints, though.

This study served as a pilot for the analysis of pSS metabolic biomarkers. The sample size should be increased in future research, and any putative metabolite biomarkers should be

validated using the targeted manner. Further research should also increase the number of pSS patient samples, improve the metabolite profiles of pSS's saliva, track changes in these biomarkers before and after treatment, and find biomarkers linked to treatment efficacy. This will hopefully provide a crucial foundation for prognosis prediction and clinical efficacy evaluation in pSS.

CHAPTER 6: CONCLUSION

According to this study, ^{1}H-NMR and LC-QTOF-MS spectroscopy can be used to quantify the amounts of metabolic components unique to a given disease in saliva. It is evident from the salivary metabolite-based diagnostics that a particular disease may be defined by a combination pattern of multiple biomarkers rather than by one. Certain biomarkers for pSS may be able to predict the condition and be used to track the disease's progression. However, further research is needed.

Within the limitations of the present study, salivary metabolomic profiling for healthy and pSS groups was successfully carried out. There was a significant difference in the salivary metabolite profile (purine) between these two groups, thus the study hypothesis was accepted, and the null hypothesis was rejected. Furthermore, this study confirms that salivary (purine) could be used as potential biomarkers in discriminating pSS from HCs.

This approach has the potential to function as a non-invasive tool that can be incorporated into the dental clinic workflow, enabling the differential diagnosis of pSS patients and monitoring disease progression. This integration will ultimately enhance patients' oral and overall health.

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