Identification of Potential Salivary Biomarkers in Primary Sjögren's Disease

A thesis submitted by

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Abstract

Objectives: Sjögren's Disease is a long-term autoimmune disease that affects lacrimal and salivary glands, and often severely affects other organ systems, such as the lungs, kidneys, and nervous system. However, the diagnosis of Sjögren's Disease is challenging and additional biomarkers of the disease are needed. We aim to uncover biomarkers that differentiate individuals with primary Sjögren's Disease (pSjD) from otherwise healthy subjects.

Methods: We obtained saliva samples from 30 patients with pSjD and 30 healthy controls. Samples were analyzed by ^{1}H-NMR (nuclear magnetic resonance) at 600 MHz. Chenomx software was used to match the experimentally obtained spectra against reference spectra to identify and quantify the concentration of metabolites. Data were normalized by sum of the metabolites per each subject, analyzed using MetaboAnalyst 5.0, and a Mann-Whitney U Test to statistically compare median values. Age-restricted data were extracted and used to explore potential biomarkers by using ROC curve analysis. $P < 0.05$ was considered significant.

Results: We quantified a total of 36 metabolites. Analysis using non-normalized data revealed increased concentration of Acetoin, Choline, Dimethylamine, Fructose, Glucose, Glycerol, Lactate, Pyruvate, Taurine, and valine in primary SjD patients as compared to healthy controls. Analysis using data normalized to the sum of metabolites showed increased concentration of Choline, Dimethylamine, Glucose, Glycerol, Lactate, Taurine, and decreased 5-Aminopentanote, Acetate, Butyrate, Fucose, and Propionate in these patients as compared to healthy individuals. Analysis

using age-restricted data indicated increased concentration of choline, glycerol, glucose, lactate and taurine, and decreased 5-aminopentanoate, acetate, butyrate and propionate in primary SjD patients as compared to healthy subjects. Receiver Operating Characteristic (ROC) curve analysis demonstrated taurine/5-aminopentanoate was a good biomarker for detecting primary SjD.

Conclusions: Patients with primary SjD exhibited significantly different metabolite concentrations as compared to healthy controls, which might be associated with either the disease or its treatment. The results indicated that patients with primary SjD have metabolic perturbances which may contribute to the pathogenesis of this disease. Analysis revealed potential biomarkers that involve the metabolism of amino acids, carbohydrates, energy production, and the microflora of the oral cavity. Among them, taurine/5-aminopentanoate was the most informative biomarker in our study.

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Voulgarelis M., Tzioufas A. G. (2010). "Pathogenetic mechanisms in the initiation and perpetuation of Sjögren's syndrome". Nature Reviews. Rheumatology. 6 (9): 529–537. doi:10.1038/nrrheum.2010.118. PMID 20683439. S2CID 8755126.

List of Abbreviations

5-APN: 5-animopentanoate.
ANA: anti-nuclear antibodies.
AUC: area under curve.
BAFF: B-cell activating factor.
CHIC: cysteine-rich hydrophobic.
DSS: sodium 2,2-dimethyl-2-silapentane-5-sulfonate.
EBV: Epstein-Barr virus.
EGF: epithelial growth factor.
FC: fold change.
FID: free induction decay.
FTX: five prime to Xist.
GC-MS: gas chromatography–mass spectrometry.
GEO2R: gene expression omnibus.
GPR: G protein-coupled receptors.
HC: healthy controls.
JAK-STAT: janus-activated kinase/signal transducer and activator of transcription.
M3R: muscarinic type 3 receptor.
mM: millimolar.
MS: mass spectroscopy.
MSGB: minor salivary gland biopsy.
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells.
NMR: nuclear magnetic resonance.
OPLS-DA: orthogonal partial least squares discriminant analysis.
PPM: parts per million.
pSjD: primary Sjögren's disease.
RF: rheumatoid factor.
RLIM: ring finger protein, LIM domain interacting.
ROC: receiver operating characteristic.
SS: Sjögren's Syndrome.
SS-A: anti-Ro.
SS-B: anti-La.
T1DM: type 1 diabetes mellitus.
TNF: tumor necrosis factor.
XIST: X-inactive specific transcript.
µl: microliter.

Chapter 1: Introduction

1.1. Sjögren's Disease

Sjögren's Disease (SjD), which is also known Sjögren's Syndrome (SS), is a chronic autoimmune disorder that takes place when the immune system attacks the fluid producing glands, such as the lacrimal and salivary glands. [1] Mononuclear cell infiltration, followed by tissue destruction, results in the development of oral and ocular dryness. [2] SjD often significantly affects other organ systems, such as the lungs, kidneys, and nervous system. [3] Primary symptoms are dryness, dry mouth, and dry eyes [4], pain and fatigue. [5] Other symptoms include dry skin, vaginal dryness, a chronic cough, numbness in the arms and legs, fatigue, muscle and joint pains, and thyroid problems. [1] SjD can occur independently of other health problems (primary Sjögren's Disease, pSjD) or because of another connective tissue disorder (secondary Sjögren's Disease), such as primary biliary cirrhosis. [6] Connective tissue disorder develops without detection over a period of months to years in most cases. [7]

The disease was described in 1933 by Henrik Sjögren, after whom it is named. However, descriptions of Sjögren existed already before that time. [6] Between 0.2 and 1.2% of the population is affected by Sjögren, with half having the primary form and half the secondary form. [8] The disease affects females about 9 times as often as males. [6] Though the disease most commonly starts in middle age, it can affect people at any age. [4][6] Among those with SjD but without other autoimmune

disorders, life expectancy is unchanged. [8]

The pathogenic mechanisms of SjD have not been fully elucidated, but in the presence of a susceptible genetic background, both environmental and hormonal factors are thought to be capable of triggering this autoimmune exocrinopathy. [9]

Genetic factors closely associated with pSjD include human leukocyte antigen type-DR (HLA-DR) allele subtypes and several specific gene polymorphisms. Zhang *et al*. analyzed salivary gland genome-wide expression profiles of pSjD patients and non-diabetic controls; 379 differentially expressed genes were found to be involved in the pathogenesis of pSjD. 300 genes that were significantly upregulated, were enriched with Gene Ontology terms of autoimmune response. [68]

Based on the predominance of females in pSjD, the X chromosome has attracted extensive attention. Numerous X chromosome genes associated in SjD pathogenesis can be modulated by transcription factors, differentially methylated, and overexpressed in SjD patients. For example, Mougeot *et al*. used the SS GEO2R (an interactive web tool) gene dataset and identified 58 X chromosome genes that were upregulated. [69][70] They found that X-inactive specific transcript (XIST), whose high expression is a known pathogenic SjD biomarker in male patients, [76] and its cis-regulatory factors, such as cysteine-rich hydrophobic (CHIC1), Five prime to Xist (FTX), and Ring Finger Protein, LIM Domain Interacting (RLIM), were upregulated. [69][70]

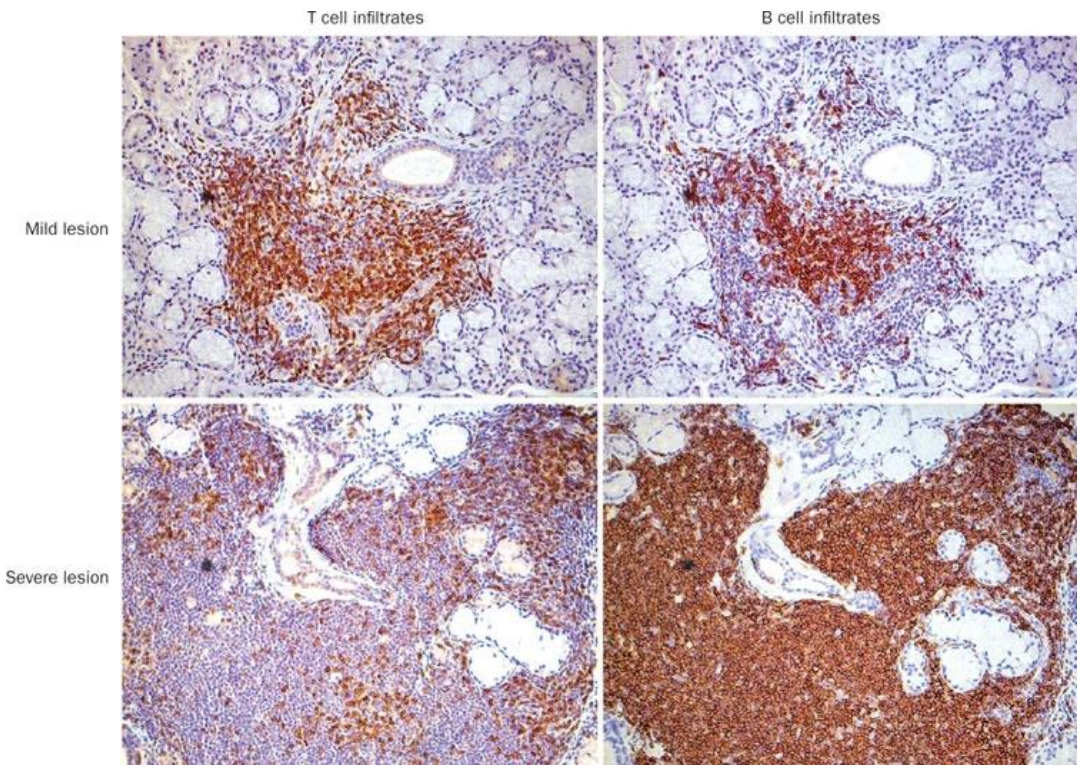


Figure 1.1: A typical inflammatory characteristic of SjD is cellular infiltration around the ducts of the salivary glands. Activated T cells prevail in mild lesions, whereas in severe lesions, B cells predominate. T cells were analyzed by rabbit-polyclonal antibodies to human CD3. The mouse monoclonal antibodies to human CD20 were used to examine the presence of B cells, original magnification ×200. [10] Reprinted with permission from Voulgarelis M., Tzioufas A. G. (2010). "Pathogenetic mechanisms in the initiation and perpetuation of Sjögren's syndrome". Nature Reviews. Rheumatology. 6 (9): 529–537. doi:10.1038/nrrheum.2010.118. PMID 20683439. S2CID 8755126.

Environmental factors, such as viral infection, are important triggers of pSjD. Virus-induced autoimmunity can be caused by various mechanisms, for instance, epitope spreading, bystander activation, molecular mimicry, and infected B-cell immortalization. [71] Sanosyan *et al*. reported that Epstein-Barr virus (EBV) is highly expressed in exocrine gland biopsies from nearly all pSjD patients and is highly linked with anti SSA/B autoantibodies and markers of B-cell activation. This suggests a potential link between EBV, B-cell activation and pSjD etiology.[72] Viral infection also causes activation of the autoimmune response, which inhibits the development of

the immune response. [71]

Cellular infiltration of epithelial cells is a significant characteristic of the initiation and progression of immune and inflammatory responses in pSjD (Figure 1.1). Under the influence of genetic susceptibility and environmental factors, key signaling pathways such as Janus-activated kinase/signal transducer and activator of transcription (JAK-STAT) and epithelial growth factor (EGF) signaling are activated in epithelial cells. This leads to excessive accumulation and activation of immune cells, such as dendritic cells, B cells and T cells. With the help of chemokines and adhesion factors, these immune cells migrate to the gland and express various pro-inflammatory factors that, in turn, activate neighboring epithelial cells. [73] An accumulation of dendritic cells can produce high levels of interferon (IFN)-α, which stimulates epithelial cells, T cells, and dendritic cells to produce B-cell activating factor (BAFF). BAFF stimulates the maturation of abnormal B cells in lymphogenic centers and the production of autoantibodies, which triggers autoimmunity response.

1.2. Potential of Biomarkers for Diagnosis

Dry mouth and oral dryness are caused by a decrease in saliva production from the salivary glands including parotid, submandibular and sublingual glands. Diagnosing SjD is complicated by the range of symptoms that a patient may express, and the similarity between symptoms of SjD and those of other conditions such as rheumatoid

arthritis and lupus. Also, patients with SjD symptoms approach different specialties for treatment, which can make diagnosis difficult. Because dry eyes and dry mouth are very common symptoms, and frequently happen in people over 40, people who are affected may believe that the symptoms are age-related, and thus they may pay no attention to them. In addition, some medications such as sex steroids cause similar symptoms. [11][12]

Disease diagnosis is aided by biomarkers. Biomarkers are not only quantitative measures that allow for more precise diagnosis, but also can be used to assess the disease process, and to monitor the response to treatment. In fact, biomarkers can be considered as the basis of therapy. By definition, a biomarker can be considered as "a characteristic objectively measured and assessed as an indicator of a normal biological process, a pathogenic process, or a pharmacological response to a therapeutic intervention". [26] There are many examples of biomarkers in SjD. The most obvious are in serum (e.g., autoantibodies and cytokines) and in DNA (determined using genetic analysis and/or genome-wide association studies), as well as in cells or at the cellular level (determined by different phenotypes or properties/function of the cells). Tissue reactions, including focal inflammation and germinal center reactions, are good examples of biomarkers. Specifically in SjD, very attractive sources of biomarkers are saliva or tears, directly produced from the target organs. [27] Anti-nuclear antibodies (ANA) [36] and rheumatoid factor (RF) are also parameters normally measured in SjD as a clinical and diagnostic tool. [37-39] Validated biomarkers are important for providing rapid and accurate diagnosis, as well

as patient classification, treatment, and follow-up. Peripheral blood samples (serum/plasma) are readily available and therefore they are the most obvious biological material when seeking biomarkers. In 2001, Jonsson *et al* published a list of potential biomarkers [28], which together with the recent reviews by Tong *et al* [29] and Fayyaz *et al* [30], provide a good overview of the SjD-related biomarkers, such as anti-Ro and anti La.

To determine whether the salivary glands are functioning adequately, a test of salivary flow-rate can be performed. [12] Other objective tests used in diagnosis include blood, eyes, and dental tests. **1)** Blood test is used to confirm marker antibodies: SS-A (or Ro) and SS-B (or La). 70% of Sjögren's patients are positive for SS-A and 40% are positive for SS-B. Autoantibodies to the autoantigens Ro/SSA and La/SSB are the most important biomarkers identified to date and have been included in the classification criteria for pSjD. [31-35] **2)** Eye test (Schirmer test) is used to measure tear production, and Rose Bengal and Lissamine Green test is used to examine the eye surface for dry spots. **3)** Dental Tests include the measurement of salivary flow, and Minor Salivary Gland Biopsy (MSGB), which is commonly referred to as the "lip biopsy". MSGB is also known as a labial salivary gland biopsy. MSGB has played an important role in the diagnosis and classification of SjD since it was first described more than 40 years ago. [49,50]. A positive MSGB strongly supports a Sjögren's diagnosis. [13] Currently, biopsy remains the best method for diagnosing the salivary gland component of SjD, primarily because of its high specificity (86.2%). Recent evidence suggests that MSGB may also provide

prognostic and stratification of patients with SjD [52-54].

In recent years, additional biomarkers have been found as promising diagnostic markers. Muscarinic type 3 receptor (M3R) is one of the new promising biomarkers with a direct biological and functional link to exocrine secretion. [40-46] Antibodies produced against M3R have the potential to inhibit salivary secretion. Another very interesting aspect is that biomarkers/diagnostic markers may be present many years before the appearance of clinical symptoms. For example, anti-Ro and anti-La immunoglobulins may be detected up to 18-20 years before the beginning of symptoms suggesting that early diagnosis and monitoring of pre-SS is possible. [47,48] The potential benefit of early diagnostic testing may be the start of an early treatment, which will result in preventing further substantial gland tissue damage.

1.3. Salivary metabolites

Metabolites, which are important indicators of physiological or pathological states, can also provide information for identifying early and differential markers of disease, and help in understanding the initiation of a disease and its progression. [55] In the era of personalized medicine, new biomarkers are needed to diagnose SjD early, define different disease subgroups, guide treatment, monitor disease progression, regression, and the clinical management of patients. [67]

Salivary metabolomics has been relatively understudied compared to blood or urine

biofluids. The growing interest in salivary metabolomics in the last decade, [16, 17] not only to find biomarkers of oral lesions, [18-21] but also some other diseases such as T1DM [22] and dementia. [23, 24] The increase interest in using saliva for disease research is influenced by the simplicity and non-invasiveness of saliva sample collection, both of which improve compliance in the elderly and in children.

Overall, saliva metabolomics has become a useful approach for identifying biomarkers for oral squamous cell carcinoma, [56,57] periodontal diseases, [58] oral lichen planus, [59] oral leukoplakia [60] and cardiovascular disease. [61] In addition, a few metabolomics studies have recently reported salivary biomarkers for pSjD diagnosis. [62-64] Mikkonen *et al*. and Herrala *et al*. used NMR spectroscopy to analyze the saliva metabolome from 14 female pSjD patients, and the levels of choline, taurine, alanine, glycine, phenylalanine, and proline were found higher in patients with pSjD than in controls. [63,64] In another study, the saliva samples of 12 pSjD patients were evaluated by gas chromatography–mass spectrometry (GC–MS), and glycine, tyrosine, uric acid and fucose were found to be downregulated. [62] Salivary gland dysfunction in primary pSjD patients unavoidably leads to changes in salivary components, and the pathogenesis of the disease can be inferred from changes in saliva. Many previous studies have focused on saliva proteomic, transcriptomic, and genomic biomarkers for pSjD; [65,66] however, a sensitive and specific biomarker in this fluid source has not yet been available. In the above-mentioned studies, the sample sizes were under 20. It is likely that a larger sample size, such as the one in this study, will more accurately represent the population value,

whereas smaller samples could be off the mark in either direction – towards or away from the population value. [86]

1.4. Nuclear Magnetic Resonance (NMR) Spectroscopy

Metabolomics is a powerful and sensitive method for assessing metabolism. That can be used to evaluate biochemical irregularities and differences in metabolism among various conditions. The method identifies compounds and the relative concentrations of these compounds within biological samples. A wide variety of biological samples can be used, such as blood, urine, cells, saliva and extracts from isolated perfused organs or tissues. The major technologies used for metabolomics are NMR and mass spectroscopy (MS). NMR spectroscopy is based on the premise that when a nucleus is placed in a magnetic field, a signal can be produced when the ground state is stimulated to the excited state. Nuclei of different chemical groups will resonate at different frequencies. The appearance of resonances at different frequencies, for a particular nucleus, is called a chemical shift. The basic concept of chemical shifts in NMR is that nuclei are associated with different bonds and different electron clouds. The electrons cause local magnetic fields that change the resonance frequency. NMR signals may have different number of peaks (the number of lines). This is called the splitting of the signal or multiplicity. Signal splitting is arguably the most unique and important feature that makes NMR spectroscopy a comprehensive tool in structure determination. The signal splitting is caused by the hydrogens on

the same carbon or on the neighboring carbons. Only nonequivalent protons split the signal of given proton(s). One adjacent proton splits an NMR signal into a doublet peak and two adjacent protons split the signal into a triplet peak. After obtaining the NMR spectra of a biological sample, a database containing approximate peak positions of common organic compounds is used for matching known substances to the experimentally obtained peaks. [25]

In comparison to MS, an important disadvantage of ^{1}H-NMR is its lower sensitivity, which makes it less suitable for measuring relatively low-abundance compounds (Table 1.1). [14] In addition, NMR instruments are usually more expensive than MS. (Table 1.1). For non-targeted analysis and discovery of new biomarkers, ^{1}H-NMR is superior to MS, which is preferred in comparison to performing targeted analysis to find specific metabolites. [15]

	NMR	MS
Detected metabolites	Universal (less biased)	Specific (more biased)
Metabolite Identification	Nearly complete	Incomplete
Sensitivity	Less sensitive	More sensitive
# of compounds quantified	Dozens to 200	Several hundred
Reproducibility	Higher	Lower
Quantitation standard	Only one needed	Standard for each metabolite

Sample preparation	Minimal	Involved
Sample integrity	Non-destructive	Destructive
Cost of instrument	More expensive	Less expensive

Table 1.1: Comparison of NMR and MS key features.

Treatment for Sjögren's syndrome depends on the organ and system that is affected. Many people manage dry eyes and dry mouth symptoms that are associated with Sjögren's syndrome by using over-the-counter eye drops and drinking water more frequently. However, some people need prescription medications or even surgical treatment. For example, prescription eye drops such as cyclosporine (Restasis) or lifitegrast (Xiidra) are used to decrease eye inflammation. Drugs, such as pilocarpine (Salagen) and cevimeline (Evoxac) can help increasing the production of saliva. Hydroxychloroquine (Plaquenil), a drug designed to treat malaria, is often helpful in treating Sjögren's syndrome to treat system wide symptoms. [88]

Currently, physicians still need nearly three to five years, on average, to diagnose the occurrence of Sjögren’s disease in patients. If we could speed up the time to diagnosis of this disease, we could offer these patients early treatment. Therefore, additional biomarkers with high specificity and selectivity for this disease are needed. Our research focuses on identifying and quantifying salivary metabolites using NMR methods to differentiate individuals with pSjD from otherwise healthy subjects.

Chapter 2: Material and Methods

2.1. Saliva sample processing

We obtained saliva samples from 30 patients with pSjD and 30 healthy controls during their study visit at Tufts University School of Dental Medicine, Boston, MA (IRB STUDY00000561 and STUDY00002230., PI: Alt-Holland).

Each saliva sample was collected using a collection straw that was connected to a cryotube and placed in a 50 ml conical tube. All samples were stored in a -20^{0}C freezer until ready for processing. Samples were thawed on ice and centrifuged at 1800g for 2 mins at 4^{0}C. While the samples were spun down, two sets of Eppendorf tubes were labeled. After centrifugation, the collection devices and cryotubes with the samples were placed in a biological hood, and the straws were removed using forceps. 350µl of supernatant of the spun down saliva samples were transferred into a corresponding labeled Eppendorf tube. When the volume of a saliva sample was less than 300µl following the first centrifugation, the volume of the supernatant was determined using a pipette. ddH_2O was added to the saliva supernatant up to a total volume of 300µl. This procedure added a dilution factor to the saliva sample and needed to be taken into account when the final concentrations of metabolites were calculated. For saliva samples that were very viscous, a portion of p1000 tips was cut off to allow for a better pipetting of the saliva. 350µl samples were centrifuged at 9000g for 5mins at 4^{0}C, of which 300µl of supernatant were transferred into the second set of labeled Eppendorf tubes. 200µl of diH_2O and 56µl of a buffer

comprising 500mM phosphate 5mM DSS in D_2O + 0.02% sodium azide was then added and vortexed for 15 seconds, and 550µl samples were transferred into NMR tubes.

2.2. NMR Spectroscopy

For each saliva sample, a ^{1}H-NMR spectrum was recorded on a Bruker Avance 600 spectrometer (Bruker Corporation, Billerica, Massachusetts, U.S.), at 25^0C, using a zgesgppe pulse sequence. A total of 128 scans were acquired into 76922 data points, a spectral width of 16 ppm, 4 seconds acquisition time and a relaxation delay of 1 second. The data was exported to Chenomx for further processing (version: 9.0, Chenomx Inc. Edmonton, Canada). Chenomx is a program that allows the identification of compounds by matching the spectra of individual compounds to its reference database of spectra. Once an NMR spectrum is obtained, its analysis involves many steps, starting with the processing of the data, phase and baseline correction, peak linewidth adjustment, spectral fitting, identification and finally quantification of metabolites. Each free induction decay (FID) was multiplied by a 0.5 Hz exponential function prior to Fourier transformation. Spectra were phase corrected, and chemical shifts were referenced internally to 0.5 mM DSS (δ 0.0). Spectra were baseline corrected, and the water region (4.5 to 5.0 ppm) was not included in the analysis. Salivary metabolites corresponding to significant spectral features were identified and characterized using the chemical shift and signal splitting

patterns of metabolites reported in the literature and the reference library within Chenomx. Metabolite concentrations were determined and exported to Excel for further analysis.

2.3. Statistical analysis

Descriptive statistics was calculated, including counts and percentages for categorical demographic variables, means and standard deviations for continuous variables, and median and interquartile ranges for non-normally distributed continuous metabolite levels. Analysis was conducted on non-normalized and normalized data. For non-normalized data (metabolite concentration), the concentration of individual metabolites in the saliva sample from each study subject was analyzed. For normalized data (metabolite normalization), the concentrations of individual metabolites were divided by the sum of the concentration of all identified metabolites in the saliva sample from each study subject. In that way, data was normalized to be the percentage that each individual metabolite comprises of the overall metabolite concentration for each subject. The distribution of the data was assessed graphically with quantile-quantile plots (q-q plots) and histograms. Since the data was not normally distributed, the non-parametric Mann-Whitney U test was used to compare median metabolite levels (either concentration or normalized data) between healthy controls and primary Sjögren's disease groups.

We also created Volcano Plot, Principal Component Analysis (PCA) analysis and a Score plot of the Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) model between pSjD subjects and healthy controls using normalized data in MetaboAnalyst 5.0. Receiver Operating Characteristic (ROC) curves were made by Graph Pad Prism (Version 9.4.1, San Diego, CA, USA) and SPSJD (version 27, Armonk, NY, USA). $P < 0.05$ was considered significant.

2.4. Author Distribution

Consenting of study participants and saliva sample collection were performed by research coordinators at the clinical research center of Tufts University School of Dental Medicine. The saliva sample processing was performed by Tatiana Mendez and Dr. Addy Alt-Holland. Most of the NMR data collection was done by Tatiana Mendez, with my assistance. Dr. Sarah Pagni performed the initial statistical analysis of non-normalized and normalized metabolomics data. The profiling work, calculating metabolites' concentration and all other data analysis work in this study were done by me.

Chapter 3: Results

3.1. Subject characteristics

The demographic characteristics of the study subjects are summarized in Table 3.1. The study included 30 pSjD patients and 30 healthy controls (HC). The disease is closely related to age. [4,6] Table 3.2 shows the age analysis of the pSjD and HC groups, and Figure 3.1 shows the age distribution of individuals in these two groups (pSjD and HC).

Group	pSjD		HC	
	male	female	male	female
number	2	28	9	21
Age	43.0±18.3	63.7±8.3	38.1±18.4	36.8±14.7

Table 3.1: Demographic of the study subjects. pSjD: primary Sjögren's disease group; HC: healthy controls. Age data showed by mean ± standard error.

Group	number	mean	median	min	max
HC	30	37.2	29	23	69
pSjD	30	62.2	63	30	78
Total	60	49.7	55	23	78

Table 3.2: Age comparison between two groups. HC: healthy controls; pSjD: primary Sjögren's disease.

As we expected, there was a significant difference in median age (29 and 63, HC and pSjD, respectively) between the two study groups ($p<0.01$). Because Sjögren's

disease is mostly age dependent [4,6], the analysis included not only a set of all samples, but also a second set of samples that was restricted in age (samples from individuals who are between 30-70 years old), for which there was no significant difference in median age between groups ($p > 0.1$). In the second set, samples from 24 pSjD patients and 14 healthy controls were included.

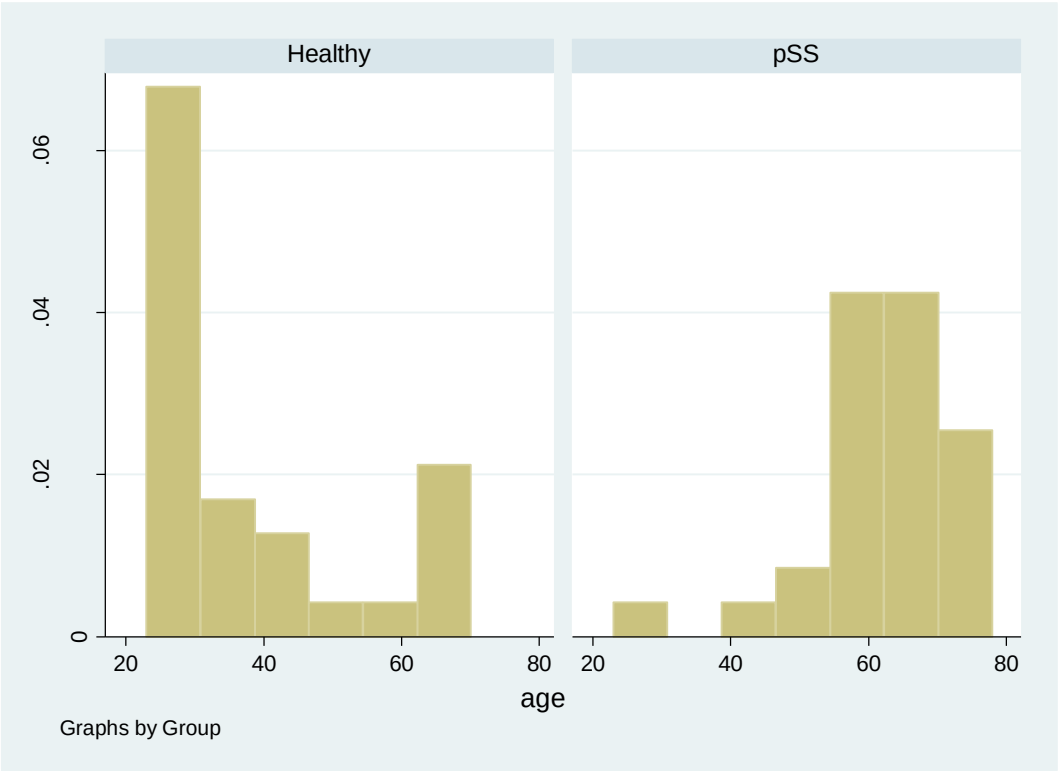


Figure 3.1: Distribution of age by Group. pSjD: primary Sjögren's disease.

Group	N	mean	p50	iqr	min	max
HC	14	50.4	49.5	28	34	69
pSjD	24	59.3	60.5	10.5	30	70
Total	38	56	59.5	14	30	70

Table 3.3: Descriptive statistics of age-restricted samples. Sample size (N), mean, median (p50), interquartile range (iqr), minimum (min), maximum (max). HC: healthy controls; pSjD: primary Sjögren's disease.

3.2. Metabolism by ^{1}H NMR spectroscopy

The ^{1}H-NMR analysis of the saliva samples yielded spectra as shown in Figure 3.2. These spectra were analyzed to identify the metabolites and to determine the concentrations compared to the 0.5 mM DSS-d6 standard, which can be seen as a peak at 0 ppm. Chenomx software was used for analysis. The concentrations were modified by the dilution factor that was required for saliva samples that had a volume of less than 300µl (Table 3.4) to yield a set of salivary metabolite concentrations (Table 3.5). We identified a total of 36 metabolites across the study subjects, as well as the sodium 2,2-dimethyl-2-silapentane-5-sulfonate-d6 (DSS-d6) NMR standard.

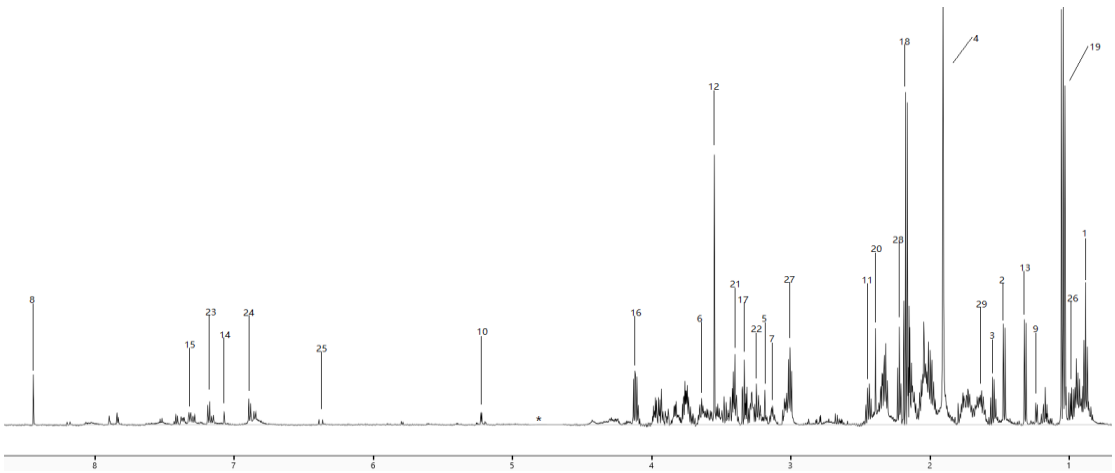


Figure 3.2: A typical ^{1}H NMR spectrum of human saliva with identified metabolites. 1,3: Butyrate; 2: Alanine; 4: Acetate; 5: Choline; 6: Ethanol; 7: Ethanolamine; 8: Formate; 9: Fucose; 10: Glucose; 11: Glutamine; 12: Glycine; 13: Lactate; 14: Histidine; 15: Phenylalanine; 16,17: Proline; 18,19: Propionate; 20: Succinate; 21,22: Taurine; 23,24: Tyrosine; 25: Urocanate; 26: Valine; 27,28,29: 5-Aminopentanoate; *: Water position.

Sample #	Comments about Saliva sample processing (when initial volume is <350µl)	Dilution Factor
1		300/556
2	Sample volume = 260µl. Added 40µl ddH_2O, for a final, total volume of 300µl.	260/556
3		300/556
4		300/556
5	Sample volume = 30µl. Added 270µl ddH_2O, for a final, total volume of 300µl.	30/556
6-21		300/556
7		300/556
8		300/556
9		300/556
10		300/556
11		300/556
12		300/556
13		300/556
14		300/556
15		300/556
16		300/556
17		300/556
18		300/556
19		300/556
20		300/556
21		300/556
22	Sample volume = 100µl and a big pellet. Added 250µl ddH_2O, for a total volume of 350µl, before second centrifugation.	100(300/350)/556
23		300/556
24	Sample volume = 100µl and a big pellet. Added 250µl ddH_2O, for a total volume of 350µl, before second centrifugation.	100(300/350)/556
25		300/556
26		300/556
27	Sample volume = 50µl and a big pellet. Added 300µl ddH_2O, for a total volume of 350µl, before second centrifugation.	50(300/350)/556

28	Sample volume = 50µl and a big pellet. Added 300µl ddH_2O, for a total volume of 350µl, before second centrifugation.	50(300/350)/556
29	Sample volume = 50µl and a big pellet. Added 300µl ddH_2O, for a total volume of 350µl, before second centrifugation.	50(300/350)/556
30	Tube contained a big pink/red pellet with supernatant. Sample was spun down, and supernatant volume = 140µl. Added 160µl ddH_2O, for a final, total volume of 300µl.	140/556
31		300/556
32		300/556
33		300/556
34		300/556
35	Sample volume = 50µl and a big pellet. Added 300µl ddH_2O, for a total volume of 350µl, before second centrifugation.	50(300/350)/556
36		300/556
37		300/556
38		300/556
39	Sample volume = 50µl. Added 250µl ddH_2O, for a final, total volume of 300µl.	50/556
40	Sample volume = 120µl. Added 130µl ddH_2O, for a final, total volume of 300µl.	120/556
41	Sample volume = 100µl. Added 200µl ddH_2O, for a final, total volume of 300µl.	100/556
42		300/556
43		300/556
44	Sample volume = 10µl. Added 290µl ddH_2O, for a final, total volume of 300µl.	10/556
45		300/556
46	Sample volume = 100µl. Added 200µl ddH_2O, for a final, total volume of 300µl.	100/556
47		300/556

48		300/556
49		300/556
50		300/556
51		300/556
52		300/556
53		300/556
54		300/556
55		300/556
56		300/556
57	Sample volume = 250µl. Added 50µl ddH_2O, for a final, total volume of 300µl.	250/556
58	Sample volume = 50µl and a big pellet. Added 300µl ddH_2O, for a total volume of 350µl, before second centrifugation.	50(300/350)/556
59		300/556
60		300/556

Table 3.4: Notes of sample processing and the resulting dilution factors. Due to reduced saliva production, samples with small volume of saliva were collected from pSjD patients.

Sample #	1	2	3	4	5	6	7	8	9	10
Group	HC	pSjD	pSjD	HC	pSjD	HC	HC	pSjD	HC	HC
5-APN	0.43	0.12	0.18	0.61	0.44	0.14	0.2	0.24	0.34	0.48
Acetate	4.12	1.63	2.53	5.78	11.27	1.47	1.85	2.67	2.96	3.94
Acetoin	0.01	0	0.02	0.02	0.12	0	0	0.01	0	0.02
Alanine	0.23	0	0.03	0.06	0.37	0.02	0.02	0.03	0.02	0.05
Butyrate	0.29	0.03	0.04	0.17	0.12	0.06	0.07	0.08	0.1	0.08
Choline	0.02	0.01	0	0.01	0.19	0.01	0	0.01	0	0.01
Citrate	0.01	0.16	0.02	0.01	0	0.1	0.03	0.03	0.06	0.01
Dimethyl sulfone	0.01	0.02	0.02	0	0.01	0	0.01	0.01	0.01	0
Dimethylamine	0	0	0.01	0	0.03	0	0.01	0	0	0.01
Ethanol	0.11	0	0.08	0.06	2.84	0.06	0.3	0.28	0.1	0.07
Ethanolamine	0.07	0.04	0.02	0.07	0.29	0.06	0.03	0.03	0.02	0.13
Formate	0.25	0.27	0.27	0.08	1.13	0.02	0.03	0.13	0.1	0.38
Fructose	0	0	0	0	0	0	0	0	0	0
Fucose	0.07	0.04	0	0.07	0	0.07	0	0.02	0.05	0.25
Glucose	0.23	0.08	0.03	0.1	0.74	0.04	0.05	0.05	0.13	0.06
Glutamine	0.35	0	0.01	0.03	0.12	0.06	0	0.02	0.04	0.08

Glycerol	0.05	0.04	0.06	0	0.15	0.03	0	0.02	0.03	0.03
Glycine	0.47	0.15	0.06	0.13	0.41	0.1	0.11	0.05	0.1	0.31
Histidine	0.08	0.03	0	0.01	0.09	0.02	0.02	0	0.02	0.07
Lactate	0.21	0.26	0.16	0.22	3.63	0.1	0.16	0.4	0.19	0.26
Maltose	0.01	0.04	0	0.03	0.15	0.01	0.02	0.04	0.03	0
Methylamine	0.01	0.01	0.01	0.02	0.05	0	0.01	0.01	0.01	0
Phenylalanine	0.06	0.03	0.01	0.01	0.05	0.01	0.01	0.01	0.01	0.02
Proline	0.77	0.06	0.04	0.12	0.42	0.13	0.12	0	0.03	0.21
Propionate	1.85	0.14	0.67	1.41	1.85	0.23	0.29	0.75	0.61	0.39
Pyruvate	0.02	0	0	0.02	0.41	0.01	0	0.01	0	0.02
Succinate	0.08	0.05	0.02	0.03	0.75	0	0.01	0.02	0.03	0.05
Sucrose	0	0	0	0	0	0	0	0	0	0
Taurine	0.13	0.15	0.11	0.12	1.24	0.03	0.09	0.14	0.15	0.11
Trimethylamine	0	0	0	0	0.01	0	0	0.01	0	0
Tyrosine	0.14	0.06	0.04	0.04	0.06	0.04	0.02	0.03	0.03	0.07
Urea	0	1.6	0.95	0.18	2.49	1	1.27	0.61	1.92	0.13
Urocanate	0.04	0	0	0	0	0	0	0	0	0
Valine	0.08	0.01	0	0.01	0.07	0	0	0.01	0.01	0.01
Xylitol	0	0.15	0	0	0	0	0	0.31	0	0.04

Table 3.5: Examples of metabolite concentrations (mM) of a representative subset of the 60 saliva samples analyzed in this study. This subset includes the analysis of saliva samples that were collected from the first 10 study subjects that were enrolled in this study. 5-APN: 5-aminopentanoate, HC: healthy controls; pSjD: primary Sjögren's disease.

3.3 Sample analysis

Having determined the metabolite concentrations, we asked whether there was a significant difference in the levels of specific metabolites between those with Sjögren's and healthy controls. Metabolomics analysis involves many steps, including pre-analytical work (e.g., sample collection and storage), analytical work (e.g., sample analysis), and data analysis (e.g., extraction and quantification of raw or normalized data). Each of these tasks may have a significant impact on quantitative results and should therefore be performed with great care. Among other factors, total sample

volume or metabolite concentrations can vary significantly from sample to sample. Therefore, it is critical to reduce or eliminate the effect of total sample variation on individual metabolite quantification.

Sample concentration or amount normalization is a well-recognized and commonly practiced analysis in metabolite measurement in the clinical field. When we compared the differences in the levels of specific metabolites between the pSjD and healthy groups, we used normalized data. When we wanted to test whether the model was good or not, which could aid in diagnosis in the clinic, non-normalized, metabolite concentration data was used. For sample normalization, we used normalization by the sum of all measured metabolites. Using the Mann-Whitney U test to compare median values, some metabolites showed significant differences in their levels ($p<0.05$) between the Sjögren's group and healthy controls group (Table 3.6 and Figure 3.3). In comparison to the HC group, the levels of choline, dimethylamine, glucose, glycerol, lactate and taurine were higher, and the levels of 5-aminopentanoate, acetate, butyrate, fucose and propionate were lower in the pSjD group. Metabolites that were included in Table 3.5 but were not included in Table 3.6 did not have significant difference in their levels between the pSjD and HC groups. We also used the Metaboanalyst 5.0 program, which is a comprehensive statistical analysis tool for metabolomics. All highly significant values from the Mann-Whitney U test that compared medians ($p<0.01$) were observed in the Volcano Plot that is shown in Figure 3.3. The Volcano plot shows the log of significance of the comparison versus the log of fold change (FC) of the mean values. In comparison to saliva samples pSjD patients, saliva

samples from healthy controls had lower levels of propionate, 5-aminopentanoate, butyrate, acetate and fucose, and higher levels of taurine, lactate, and glycerol. Next, orthogonal partial least squares discriminant analysis (OPLS-DA) demonstrated a better separation for differential metabolite selection, as shown in Figure 3.4. The x-axis shows the difference between the pSjD and HC groups, while the y-axis shows the differences within each of these groups. Permutation tests (100 times) were performed to confirm the stability and robustness of the supervised model presented in this study (intercept of Q2=0.269, $p<0.01$). OPLS-DA suggested that there were many metabolic profiling variations between the two groups (pSjD vs HC).

	Median		Mean	
Metabolites	Change	P-value	Change	P-value
5-Aminopentanoate	Down	<0.001	Down	<0.001
Acetate	Down	<0.001	Down	0.002
Butyrate	Down	<0.001	Down	<0.001
Choline	Up	0.046	/	/
Dimethylamine	Up	0.030	/	/
Fucose	Down	0.002	Down	0.002
Glucose	Up	0.007	/	/
Glycerol	Up	0.008	Up	0.008
Lactate	Up	0.001	Up	0.002
Propionate	Down	<0.001	Down	<0.001
Taurine	Up	<0.001	Up	0.001

Table 3.6: Examining differences (Sjögren's vs healthy controls) between metabolites concentrations and normalized data using Mann-Whitney U test, and the mean values is the average values from normalized data in two groups. Down means concentrations were lower in pSjD group than HC. Up means concentrations were higher in pSjD group than HC.

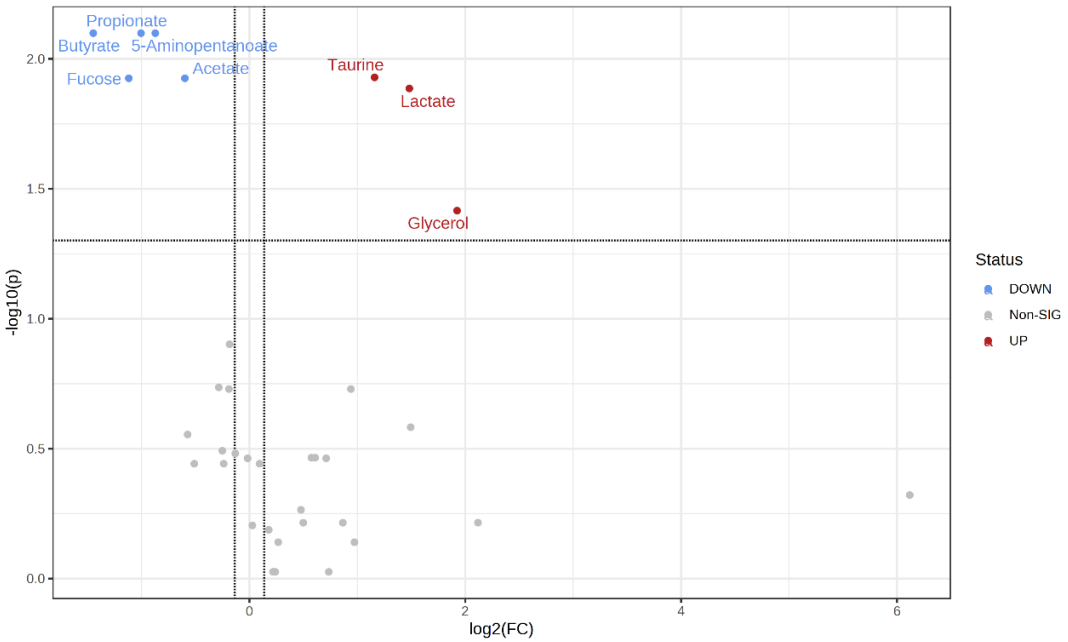


Figure 3.3: Volcano Plot of normalized data using fold change and t-test analysis to compare means of Healthy controls vs. Sjögren's disease groups. FC: fold change.

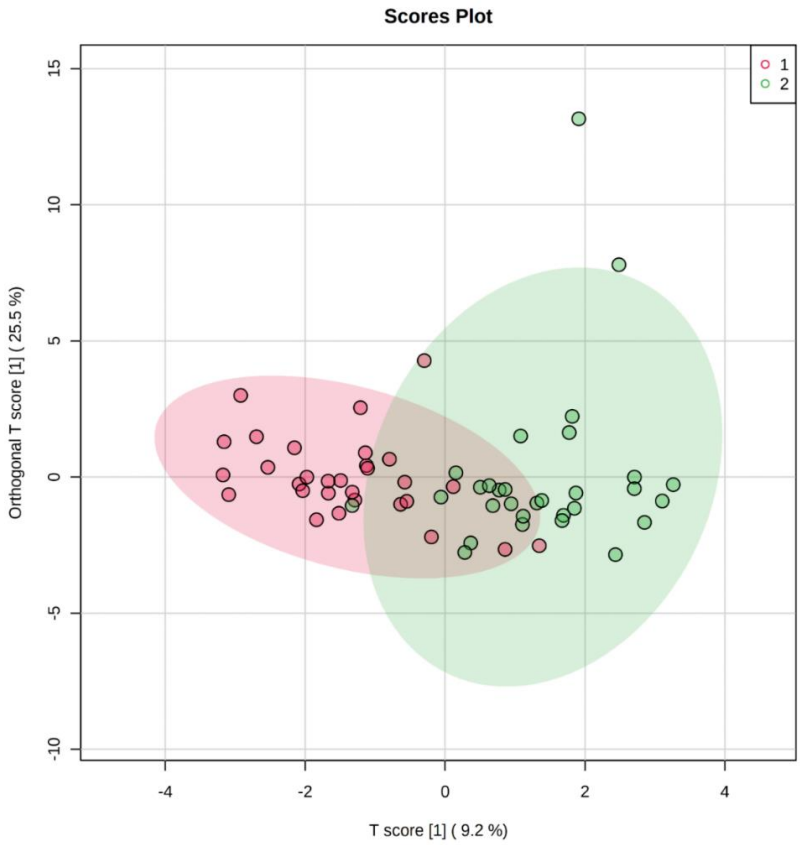
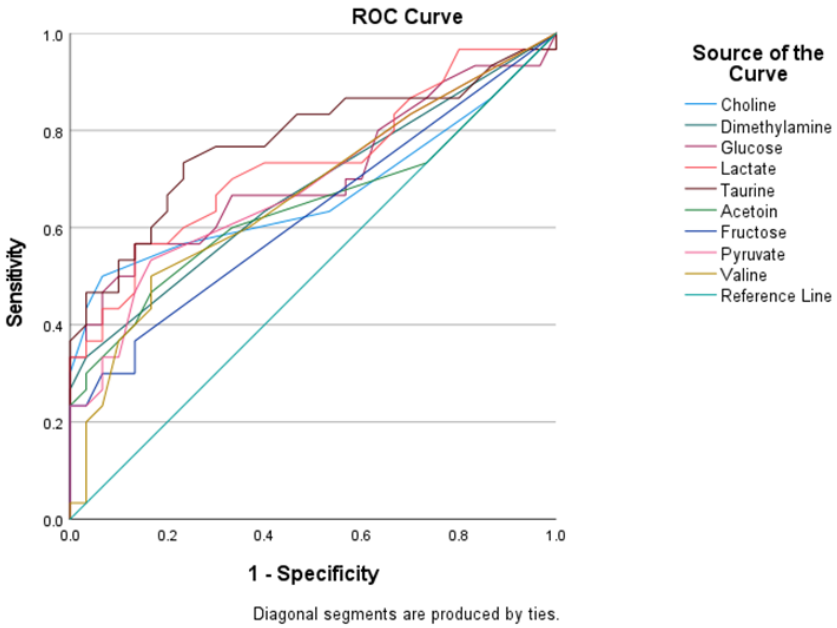


Figure 3.4: Comparison of normalized metabolomics data between pSjD subjects and HC. Score plot of the OPLS-DA model between pSjD subjects (red, 1) and HC (green, 2). HC: healthy controls; pSjD: primary Sjögren's disease.

Next, we analyzed whether those significantly changed metabolites, may serve as potential biomarkers and indicate if a saliva sample is collected from a primary SjD patient or otherwise healthy individual. This will, ultimately, allow us to predict Sjögren's disease. Receiver operating characteristic (ROC) analysis is a useful tool for assessing the performance of diagnostic tests and, more generally, the accuracy of a statistical model that classifies subjects into one of two categories, i.e., diseased or not diseased [75].

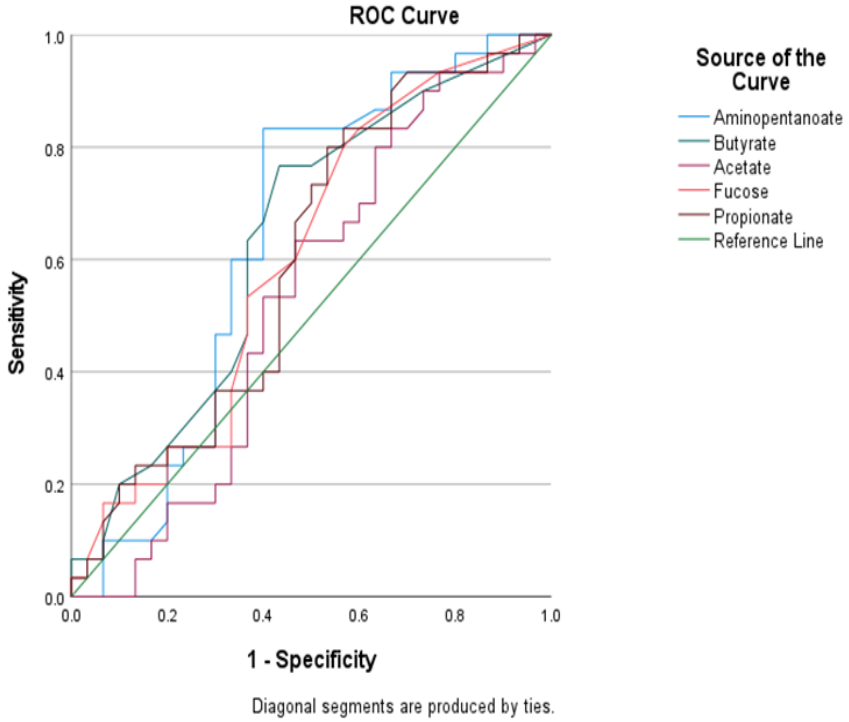


Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval Lower Bound	Asymptotic 95% Confidence Interval Upper Bound
Choline	.671	.073	.023	.527	.814
Dimethylamine	.677	.070	.018	.540	.814
Glucose	.708	.069	.006	.573	.843
Lactate	.731	.066	.002	.602	.860
Taurine	.778	.062	.000	.656	.900
Acetoin	.644	.073	.055	.501	.788

Fructose	.629	.072	.086	.487	.771
Pyruvate	.687	.069	.013	.551	.823
Valine	.669	.070	.025	.531	.807

Figure 3.5: Potential metabolic biomarkers with high concertation levels for pSjD prediction in comparison to healthy controls. ROC curve analysis for specific metabolites that were higher in saliva samples from the pSjD than HC groups. Asymptotic Sig.b: p value.



Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval Lower Bound	Upper Bound
Aminopentanoate	.642	.075	.059	.495	.788
Butyrate	.636	.073	.071	.493	.778
Acetate	.532	.077	.673	.381	.682
Fucose	.605	.074	.162	.460	.750
Propionate	.601	.074	.179	.455	.747

Figure 3.6: Potential metabolic biomarkers with low concentration levels for pSjD prediction in comparison to healthy controls. ROC curve analysis for specific metabolites that were lower in pSjD patients than healthy controls. Asymptotic Sig.[b]: refers to a asymptotic p-value that is calculated using an approximation to the true

distribution.

First, we used the data in Table 3.5, to analyze the metabolites that were significantly increased in their levels when compared to their median values. An AUC of 0.70 to 0.80 is considered acceptable; 0.80 to 0.90 is considered excellent, and more than 0.90 is considered outstanding. [74] The area under the curve (AUC) of taurine (0.78), lactate (0.73) and glucose (0.71) were greater than 0.70 and their p-values were less than 0.05 (<0.001, 0.002 and 0.006, respectively). The AUCs for choline (0.67), dimethylamine (0.68), pyruvate (0.69) and valine (0.67) are greater than 0.60, but less than 0.70, and the p-values were less than 0.05 (0.023, 0.018, 0.013 and 0.025, respectively). The AUCs of acetoin (0.64) and fructose (0.63) were greater than 0.60, and the p-values were greater than 0.05 (0.055 and 0.086, respectively) (Figure 3.5). Next, we used the data in Table 3.6, to perform ROC analysis for metabolites that were significantly decreased in their levels when compared to their median values. However, as shown in Figure 3.6, no metabolite showed significant differences and all AUC values were less than 0.7 (5-Aminopentanoate, p=0.075, AUC=0.64; butyrate, p=0.073, AUC=0.64; acetate, p=0.077, AUC=0.61; fucose, p=0.074, AUC=0.61; propionate, p=0.074, AUC=0.60).

3.4. Sample analysis with “matched” ages

Next, we performed analysis on an age-restricted data set that comprised 38 samples; 14 from healthy controls and 24 from the pSjD group (Table 3.3). As shown

in the Volcano plot in Figure 3.7, nine metabolites showed significant differences in their levels between the pSjD group and the healthy control group. In comparison to the levels of metabolites in the HC group, 5-Aminopentanoate (P<0.001), acetate (P=0.001), propionate (P<0.001) and butyrate (P=0.005) were lower in while taurine (p<0.001), choline (p=0.003), glucose (p=0.004), glycerol (p=0.005) and lactate (p=0.02) were higher in their levels in the pSjD group.

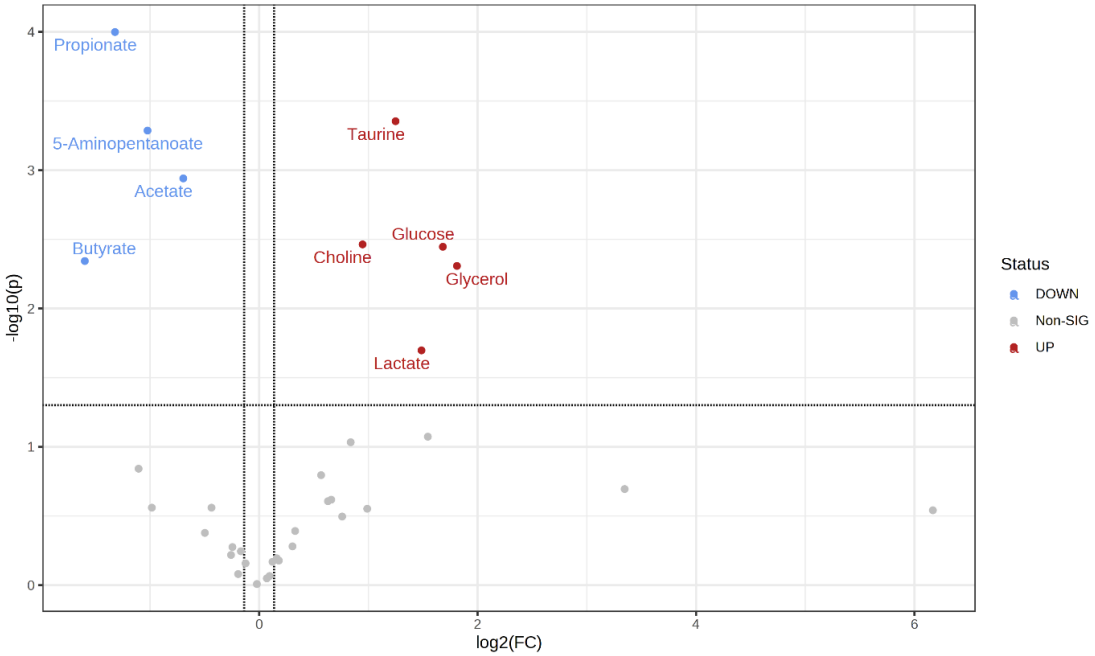
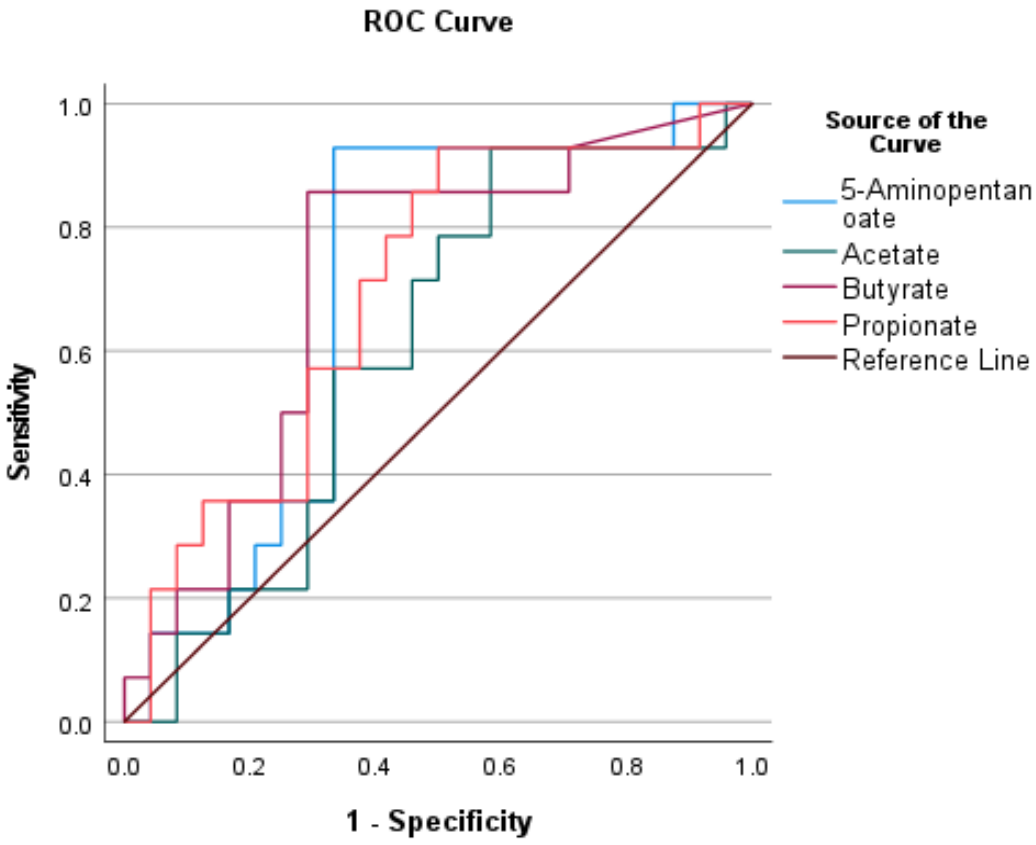


Figure 3.7: Volcano Plot, normalized and age-restricted data, using fold change and t-test analysis. FC: fold change.

Thereafter, we performed ROC curve analysis for metabolites that showed significant differences in the Volcano Plot for those that decreased (Figure 3.8) or increased (Figure 3.9) in their levels in the pSjD group relative to the HC group. For the 4 metabolites that were lower in pSjD relative to HC, butyrate (p=0.014), 5-aminopentanoate (p=0.027) and propionate (p=0.025) showed significant differences.

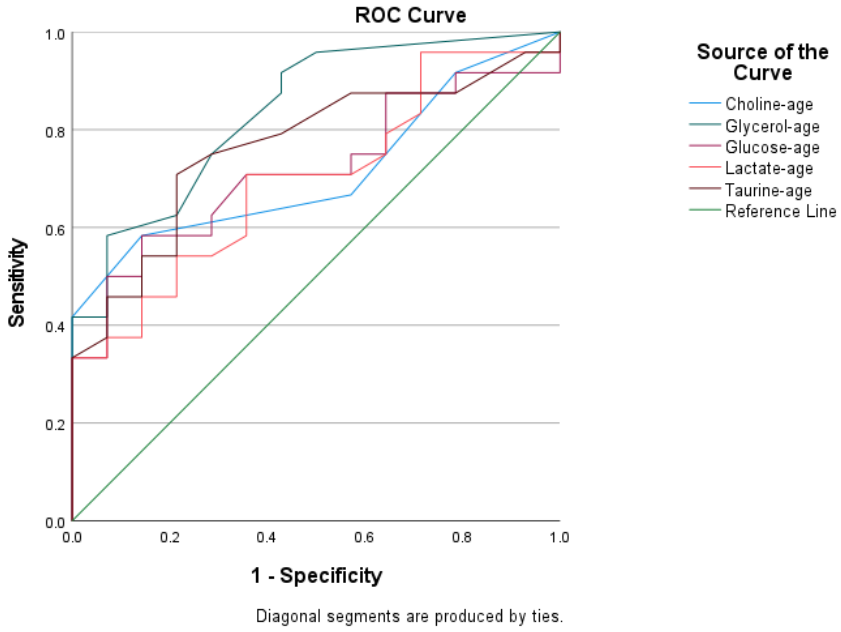
However, the level of acetate (p=0.237) did not show a significant difference. Only butyrate had an AUC greater than 0.7 (0.716). Other AUC values were less than 0.7 (5-aminopentanoate, 0.696; acetate, 0.610; propionate, 0.696). For 5 metabolites that increased in their levels, all showed significant differences (choline, p=0.029; glycerol, p=0.001; glucose, p=0.027; lactate, p=0.044; taurine; p=0.007). The AUC of glycerol was greater than 0.8 (0.838), the AUC of taurine (0.765), glucose (0.717) and choline (0.714) were greater than 0.7, and the AUC of lactate was less than 0.7 (0.698).



Area Under the ROC Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
5-Aminopentanoate	.696	.089	.027	.522	.871
Acetate	.610	.093	.237	.428	.793
Butyrate	.716	.088	.014	.544	.887
Propionate	.696	.088	.025	.524	.869

Figure 3.8: ROC curve analysis for metabolites which were lower in their levels in pSjD than in HC, non-normalized and age-restricted data, Asymptotic Sig.[b]: p value.



Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
Choline	.714	.082	.029	.553	.876
Glycerol	.838	.065	.001	.710	.966
Glucose	.717	.082	.027	.556	.879
Lactate	.698	.085	.044	.531	.865
Taurine	.765	.078	.007	.612	.918

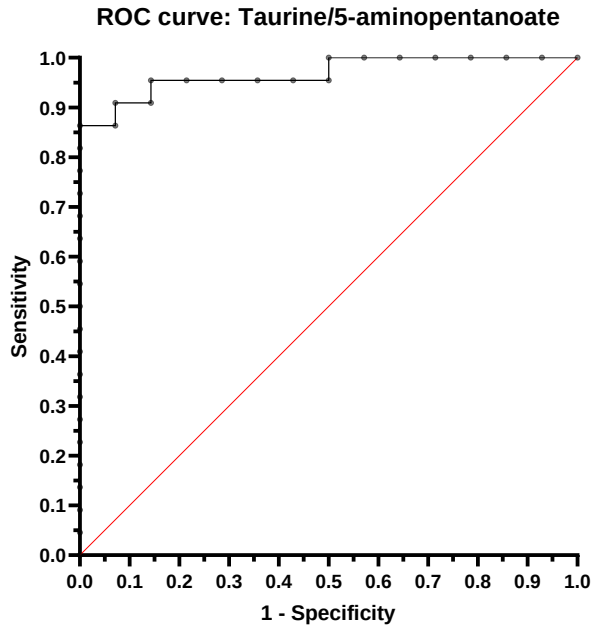
Figure 3.9: ROC curve analysis for metabolites which higher in their levels in pSjD than in HC, non-normalized and age-restricted data, Asymptotic Sig.[b]: p value.

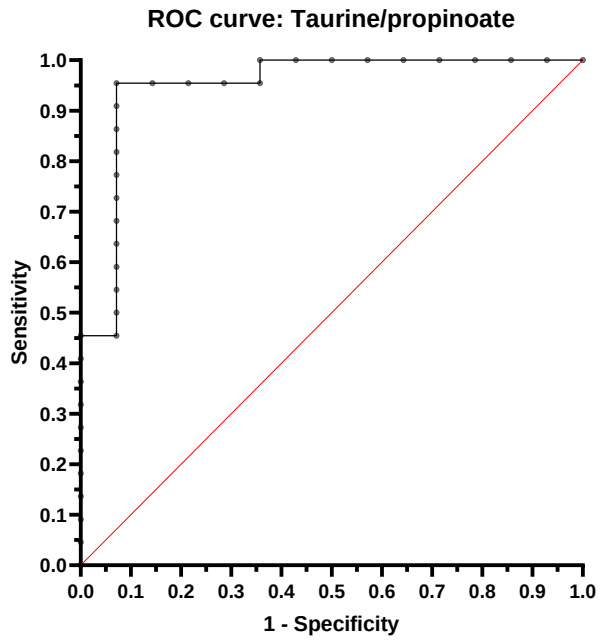
Following the completion of ROC analyses for individual metabolites, we analyzed the ratio of levels of specific metabolites that showed significant differences in the saliva samples from pSjD patients and healthy controls. We analyzed the ratio between metabolites of the 5 metabolites that were higher and the 4 metabolites that were lower in their levels in pSjD vs. HC groups for age-restricted and non-normalized data (Table 3.7). All the results showed a significant difference with AUCs greater than 0.75. The AUCs of taurine/5-aminopentanoate, taurine/propionate, glucose/butyrate, taurine/butyrate, choline/propionate, taurine/acetate and choline/5-aminopentanoate were greater than 0.9 (0.97, 0.95, 0.95, 0.94, 0.93, 0.93, 0.93 and 0.91, respectively) (Figure 3.10).

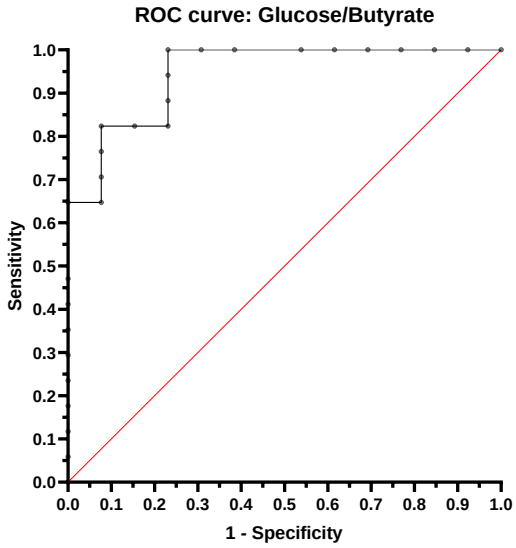
Name	P-value	AUC
Taurine/5-APN	<0.0001	0.9675
Taurine/Propionate	<0.0001	0.9481
glucose/Butyrate	<0.0001	0.9457
Taurine/Butyrate	<0.0001	0.9367
Choline/Propionate	<0.0001	0.9351
Taurine/Acetate	<0.0001	0.9256
Choline/5-APN	<0.0001	0.9026
glucose/Propionate	<0.0001	0.8994
glucose/5-APN	0.0001	0.8864
Choline/Acetate	0.0002	0.8720
Lactate/Propionate	0.0004	0.8571
Choline/Butyrate	0.0011	0.8529
glycerol/Propionate	0.0006	0.8425
glucose/Acetate	0.0007	0.8333
glycerol/Acetate	0.0011	0.8199
Lactate/Butyrate	0.0036	0.8145

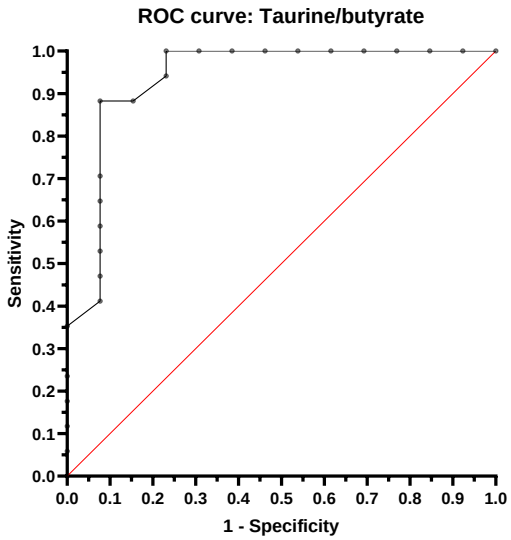
Lactate/5-APN	0.0021	0.8084
glycerol/Butyrate	0.0079	0.8021
Lactate/Acetate	0.0065	0.7679
glycerol/5-APN	0.0121	0.7570

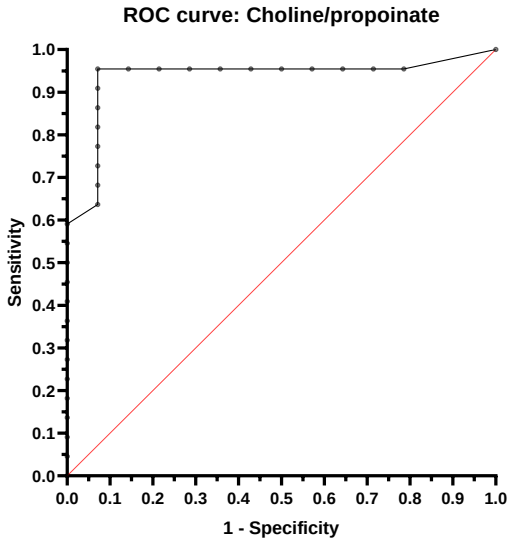
Table 3.7: ROC curve analysis of specific metabolite ratios of non-normalized and age-restricted data. 5-APN=5-aminopentanoate.






ROC curve: Glucose/Butyrate
Sensitivity
1 - Specificity
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0


ROC curve: Taurine/butyrate
Sensitivity
1 - Specificity
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0


ROC curve: Choline/propoinate
Sensitivity
1 - Specificity
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

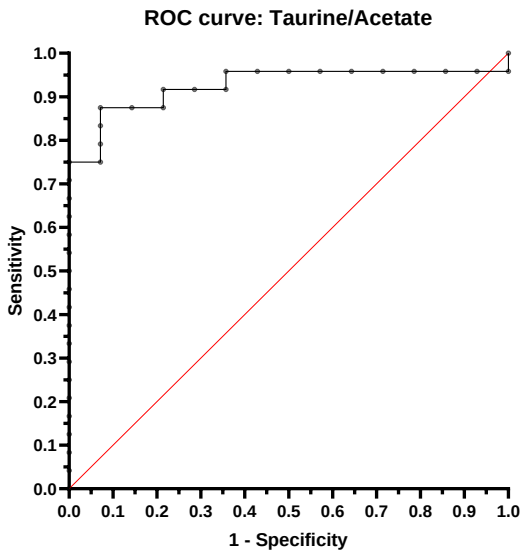


Figure 3.10: ROC curve analysis of metabolite ratios, using age-restricted, non-normalized data. Red line: reference line.

As previously mentioned, an AUC of 0.70 to 0.80 is considered acceptable; 0.80 to 0.90 is considered excellent, and more than 0.90 is considered outstanding. [74] Using these AUC values as a guide in this study, the metabolites, taurine (AUC=0.77), glucose (AUC=0.72), choline (AUC=0.71) and butyrate (AUC=0.72) could be considered as an acceptable model for the diagnosis of primary Sjögren's disease, while glycerol (AUC=0.84) can be considered as an excellent test. The analysis of metabolites ratio, taurine/5-aminopentanoate (AUC=0.97), taurine/propionate (AUC=0.95), glucose/butyrate (AUC=0.95), taurine/butyrate (AUC=0.94), choline/propionate (AUC=0.94), taurine/5-aminopentanoate (AUC=0.93) and choline/5-aminopentanoate (AUC0.90) can be considered to be outstanding diagnostic criteria for primary Sjögren's disease.

3.5. Author Distribution

Figure 3.1 was done by Dr. Sarah Pagni and the dilution factor table was done in Dr. Alt-Holland's lab. All other figures and tables were done by me. The initial data analysis was done by Dr. Sarah Pagni. Other data analysis were done by me.

Chapter 4: Discussion

This thesis study compared the metabolomics data from saliva samples that were collected from individuals with primary Sjögren's disease and otherwise healthy subjects. The metabolic profiles of these samples were analyzed and discussed in this thesis. We were able to distinguish the pSjD group from the control group in our pilot investigation of pSjD saliva metabolic profiling, and panels of metabolites identified in this study may become useful for pSjD diagnosis. The aim of the comparison was to discover biomarkers that distinguish patients with primary Sjögren's disease from other healthy subjects, in order to accelerate the time to diagnosis of this disease and, in turn, better disease management and monitoring of patients' response to treatment.

Using the saliva samples processed by Dr. Alt-Holland's lab in the School of Dental Medicine of Tufts University, the concentrations of metabolites were investigated to find the differences between the two groups (pSjD and healthy controls) from which they were collected. In my thesis, normalized metabolite raw data was used for comparisons between the two groups, while non-normalized data was used to evaluate the model of diagnosis. In this study, we conducted a comprehensive characterization of the saliva metabolome of 30 pSjD patients and 30 healthy subjects. Our results suggest that the saliva metabolome can be used to differentiate pSjD patients from healthy controls. Sjögren's disease is age dependent and is most often diagnosed between 30 and 50 years of age. [4] Following the analysis of the samples from all study participants, we selected a set of samples that was from individuals between 30-

70 years old only. This age-restricted set included metabolomics data of 24 saliva samples from the pSjD group and 14 samples from the healthy controls group), for which there was no significant difference in the median age between groups ($p > 0.1$). This approach confirms statistically significant differences between the levels of specific salivary metabolites in the two groups (pSjD vs HC) and suggests that ROC curve analysis may present as a good model for clinical diagnosis of pSjD.

This study demonstrated that it is possible to measure the levels of disease specific metabolic components of saliva using ^{1}H-NMR spectroscopy, which is consistent with a previous study. [64] In the diagnostics based on salivary metabolites, it is clear that a combination pattern of several biomarkers, rather than a single metabolite may best point on a specific disease. [79] In this study, we conducted ROC curve analysis of multiple ratios between metabolites, which was not done in previous studies. Our study population included 30 pSjD subjects out of a total of 60 individuals. In the age-restricted metabolomics data set from 24 age-matched pSjD patients and HCs was analyzed. In both cases, the pSjD population in this study was large, in comparison to previously published studies. [63, 64] While 34 pSjD patients were included in the study of Li *et al*.,[80] the highest AUC was seen in phenylalanine/alanine ratio (AUC=0.85), while our study revealed a higher AUC for taurine/5-aminopentanoate ratio (AUC=0.97) was found in our study.

Saliva is produced primarily by the major salivary glands, and the impaired secretion of the salivary glands can be described by the metabolites of saliva, which is

an excellent medium for clinical diagnostics. The whole saliva is a complex fluid containing a variety of substances, and changes in the levels of these substances are related to the pathogenesis of various diseases. However, the function of the salivary glands in patients with pSjD is severely weakened, which results in a significant reduction of saliva production and secretion in these patients. This was detected also in our study, as some of the samples from pSjD patients had a very small volume (Table 3.4) and required a large dilution factor (up to 12.5) with water.

This discussion herein focuses only on normalized and age-restricted data of the metabolites, **taurine, choline, lactate, glycerol and glucose**, which were elevated in their levels in pSjD group relative to the healthy control group. Taurine is closely involved in oxidative stress and plays an important role in cellular responses to osmotic stress, regulating volume changes, and the final composition of saliva through sodium flux. The primary role of taurine in the immune system relates to its antioxidant effect. Taurine is known to accumulate in inflammatory lesions. Changes in the levels of taurine were demonstrated to constitute a protective effect mediated by the regeneration of inflammatory and antioxidant properties in periodontal tissues. [63] Choline, a quaternary amine, is an essential nutrient that is predominantly supplied through diet. Choline-containing metabolites are important constituents of the phospholipid metabolism of cell membranes and are associated with malignant transformation. In this study, the levels of taurine and choline significantly elevated in the pSjD group in comparison to the HC group, which is consistent with a previous study. [63] The levels of Choline and taurine may be used as metabolites of interest

when following the progression of pSjD, as well as for monitoring tissue damage because choline is linked to cancer metabolism, [77] and taurine is closely involved in oxidative stress [78].

The metabolites lactate and glucose are related to energy production and carbohydrate metabolism. Glycerol is used in allergen immunotherapies, including cough syrups, elixirs and expectorants, toothpaste, mouthwashes, skin and hair care products, shaving cream, soaps, and water-based lubricants. Patients that are diagnosed with pSjD might extensively use glycerol-based lip balm to avoid dry lips, which may explain its elevated level in the pSjD group when compared to the HC group. Of note, the concentration of glycerol was below the threshold of measurement in the profiling step in this study. This potential measurement limitation might give a false negative result for glycerol.

In this study, we found a decrease in the levels of **5-aminopentanoate, butyrate, propionate and acetate** in pSjD group, when compared to the HC group. Of these metabolites, acetate has two carbon atoms, propionate has three, butyrate has four and 5-aminopentanoate has five carbon atoms, all with terminal carboxylate groups. Butyrate, a gut microbiota-derived metabolite, is a crucial metabolite that provides energy for colonic epithelial cells to maintain intestinal barrier functions. Moreover, butyrate acts as an anti-inflammatory molecule, capable of inhibiting NF-κB activation in the host immune cells by binding to G protein-coupled receptors (GPR43 and GPR41), thereby blocking inflammatory responses and suppressing TNF-alpha

and IL-6 release. [36] Since Sjögren's disease is a chronic autoimmune disorder, reduction of butyrate might cause pSjD patients to become more prone to have an autoimmune disorder. Importantly, our ROC analysis demonstrated that the level of butyrate was more than 70% (71.6) lower in saliva samples from pSjD patients (Figure 3.8). The metabolite 5-aminopentanoate is present in human saliva, with a tendency to be elevated in patients with chronic periodontitis. [87] Therefore, decreased concentration of 5-aminopentanoate may reflect a dry mouth in pSjD patients.

Based on the Mann-Whitney U tests that compared median values of salivary metabolites in this study, the level of **dimethylamine**, which is associated with gut microbiome metabolism, was higher in the pSjD group than in the control group (Table 3.5). However, this finding was not revealed in the analysis of age-restricted data, which indicates that the level of dimethylamine may be age-related. Aging has been reported to lead to elevated concentration of dimethylamine in urine, and this increase has been postulated to be due to impairment of renal function [81,82] and/or impairment of muscle composition. [83] Urinary dimethylamine was also found elevated in patients with hypertension and cardiac dysfunction. [84] However, we did not find a report on changes in salivary dimethylamine levels. Another metabolite, choline, has been reported to be lower in older individuals, [85] but when examining the median values in this study, the pSjD group had a higher level of choline in comparison to the HC group. Interestingly, there was no significant difference between these two groups when we compared median values, whereas when we

compared mean values of two groups, choline had significant differences. We hypothesize that the rise in salivary choline concentrations in pSjD patients was offset by a decrease in choline concentrations with increasing age, which was seen in our age-restricted data analysis.

Because the clinical presentation of Sjögren's disease varies a great deal and there is no ONE test that can diagnose the disease early, multiple tests are used in the diagnosis of Sjögren's. Blood tests such as anti-SSA autoantibodies are positive in only 30-70% of Sjögren's patients. They are not considered completely specific to the disease, since they are also found in 30% of lupus patients. [1] The MSGB test reflects the salivary gland component of Sjögren's, which is just one aspect of this multi-system disease. Salivary sicca is present in most Sjögren's patients, but may not be present in the early stage of the disease, or in patients with involvement of organ systems (especially neurological). Importantly, since there is no ONE objective, conclusive and early diagnostic test, a negative finding does not rule out the presence of Sjögren's disease. As for the MSGB test, technical variability can be found between different providers. Some patients refuse the procedure, especially when they hear from others who experienced a painful MSGB. [13]

Saliva samples are not invasive which would help people avoid pain and be compliant with collecting a saliva sample. The ROC curve data analysis that was conducted in this study indicated that the AUC of the ratio between the metabolites, taurine and 5-aminopentanoate was 0.97 with a p value of less than 0.001 (Figure

3.10). This indicates a 97% chance that a healthy individual can be distinguished from a pSjD patient. Yet, several factors and limitations have emerged during the study and analysis, and can potentially affect the study's outcomes. Although study participants were instructed not to eat, drink or smoke for up to two hours before participating in this study, confounding variables, such as consumption of food and medications like pilocarpine (Salagen) and cevimeline (Evoxac), might affect the composition and concentration of metabolites in saliva samples. Another factor which may increase the error in metabolite measurement is the thick saliva of some pSjD patients, which challenged an accurate pipetting. Occasionally, the concentration of metabolites, such as glycerol that is described above, were too low to measure, which makes the accurate metabolic profiling more challenging. While this study included 30 pSjD patients, an even larger sample size, as mentioned above, would more accurately represent the population [86]. As Sjögren's disease is age- and gender dependent disease, [4,6] our future efforts will involve expanding the number of pSjD patients and healthy controls in our studies to ensure the analysis of salivary samples that are accurately age- and gender-matched.

Chapter 5: Bibliography

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