Metabolomic Profiling of Serum and Saliva from Melanoma Patients

Bachelor thesis

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Melanoomiga patsientide seerumi ja sülje metaboloomiline profileerimine

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Declaration

I hereby declare that I have written this thesis independently and the thesis has not previously been submitted for defence. All works and major viewpoints of the other authors, data from sources of literature and elsewhere used for writing this paper have been properly cited.

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Abbreviations

AU	arbitrary units
CSI	chemical shape indicator
DSS-d6	3-(trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt
EDTA	ethylenediaminetetraacetic acid
IS	internal standard
MQ Water	water purified using a Millipore Milli-Q lab water system
NMR	nuclear magnetic resonance
ppm	parts per million
RCF	relative centrifugal force
UV	ultraviolet
WMS	whole-mouth saliva

Introduction

Metabolomics, the comprehensive study of metabolites within a biological system, offers a powerful approach to understanding the body's biochemical responses to various conditions, including cancer. By analysing the concentration fluctuations of specific metabolites, researchers can gain insights into metabolic changes that occur during disease progression (Nicholson, J., Lindon, J.2008).

Melanoma is a type of skin cancer originating in melanocytes, the cells responsible for producing melanin, the pigment that gives skin its colour. Alarmingly, the incidence of melanoma is rising. Its ability to spread to other parts of the body makes early detection crucial for successful treatment. The effectiveness of different treatments varies for patients, so it is important to develop methods to assess the effects of the treatment applied to a particular patient as early as possible to improve treatment outcomes (Dougherty et al, 2024).

Nuclear magnetic resonance (NMR) spectroscopy is a non-invasive analytical technique that provides comprehensive spectral data for all the metabolites present within a sample. The ability to obtain in-depth insights into the metabolite composition makes NMR spectroscopy a valuable and efficient approach in research and diagnostic applications (Nagana Gowda & Raftery, 2021).

The aim of this study was to ascertain metabolomic profiles of serum and saliva from melanoma patients and compare them to those of healthy control subjects. Our approach involves collecting body fluid samples and obtaining NMR spectra to quantify metabolite concentrations. We hypothesized that the melanoma`s presence can be observed by measuring concentrations of specific metabolites and detecting changes in the metabolomic profile. This work paves the way for future efforts to identify biomarkers indicative of disease progression or for treatment monitoring.

1. Overview of the Literature

1.1. Melanoma

Melanoma is a type of skin cancer that originates in melanocytes, the cells responsible for producing melanin, the pigment that gives skin its colour. It is considered the most dangerous form of skin cancer due to its ability to spread to other parts of the body (metastasize) if not detected early (Dougherty et al, 2024).

Risk factors for melanoma include excessive exposure to ultraviolet (UV) light from the sun or tanning beds, a history of sunburns, having a fair complexion, numerous moles or atypical moles, a family history of melanoma, and a weakened immune system. The incidence of melanoma is rising, particularly among younger individuals (Dougherty et al, 2024).

Early detection of melanoma is critical, as it significantly improves the chances of successful treatment. Warning signs include changes in the size, shape, or colour of existing moles, the appearance of new pigmented or unusual-looking growths on the skin, and any skin lesion that changes, itches, or bleeds (Dougherty et al, 2024; Boutros et al, 2024). The standard method to evaluate a skin growth to rule out melanoma is by biopsy followed by histopathological examination. The challenge lies in identifying the lesions that have the highest probability for being melanoma. Such lesions should be biopsied, and their histopathology appropriately evaluated at the earliest possible time in their development (Rigel et al, 2010).

Melanoma can affect individuals across all age groups; however, the risk significantly increases with age. Older adults are at a higher risk of developing melanoma. This heightened risk is due to cumulative exposure to UV radiation over a lifetime, along with age-related changes in skin biology. Studies have shown that melanoma incidence rates are highest among older populations, with men over 50 being particularly vulnerable (Dougherty et al, 2024). It is crucial to carefully examine the process of treatment and assess the response to therapy, as each individual's reaction is inherently unique. Understanding this variability is essential for tailoring medical interventions to achieve optimal outcomes for every patient (Boutros et al 2024; Long et al 2016).

1.2. Metabolomics

Metabolomics is the comprehensive study of metabolites, which are small molecules involved in various biochemical processes within cells, tissues, and organisms. It focuses on the systematic identification, quantification, and analysis of these metabolites, providing a detailed snapshot of the metabolic state of a biological system at a given time. This field is critical in understanding how cellular processes are altered in response to genetic changes, environmental factors, or disease conditions, making it a valuable tool in biomedical research, including cancer studies (Bujak et al, 2015).

Metabolomics employs advanced analytical techniques, such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), to detect and quantify a wide

range of metabolites. The resulting data can help elucidate complex biochemical pathways, identify biomarkers for disease diagnosis and prognosis, and even monitor treatment responses (Nagana Gowda & Raftery, 2021).

Metabolites are substances with low molecular weight (<1500 Da), intermediates, and products of chemical reactions catalyzed by various enzymes in living systems. Each biofluid, such as urine, saliva, or serum, contains a unique and complex array of metabolites. The metabolome—the complete set of metabolites within a biological sample—varies significantly between different types of fluids, reflecting the distinct physiological and biochemical environments from which these fluids originate (Kiseleva et al 2022).

The sheer number of metabolites present in human biofluids presents both opportunities and challenges in metabolomics research. While comprehensive profiling could theoretically provide exhaustive insights, it is neither feasible nor necessary to monitor every metabolite due to the complexity and volume of data. Therefore, researchers typically focus on a subset of key metabolites—those that are consistently detectable and are known or suspected to be involved in specific biological pathways or disease processes.

The serum is obtained when whole blood coagulates. In a laboratory, it is common to centrifuge the coagulated blood to the bottom leaving serum above it. The main components of serum are water, various peptides, proteins, amino acids, nitrogen compounds, hormones, various salts and ions, and traces of nucleic acids, metabolites, and lipids (Kiseleva et al 2022). From serum 37 229 metabolites have been detected, 3128 of those have also been quantified (Psychogios et al 2011).

Metabolites of plasma, the liquid component of blood, where coagulation has been prevented, have also been analysed in many metabolomic studies. Plasma is obtained when anticoagulant, ethylenediaminetetraacetic acid (EDTA), heparin, or citrate, is added to whole blood and then centrifuged, to separate the cellular material from the lighter liquid layer (Kiseleva et al 2022). Metabolite content of plasma is quite similar to that of serum. They are biologically equivalent, having small concentration differences of some metabolites (Suarez-Diez M, Adam J, 2017).

Saliva is produced in the oral cavity by three pairs of major glands and around one thousand minor glands. When referring to the fluid produced upon spitting it is called whole-mouth saliva (WMS) (Al-Tarawneh et al 2011). Salivary metabolome composition is variable between individuals and also within the same individual (Maruyama Y, Nishimoto Y, Umezawa K, 2022). From saliva 886 metabolites have been detected and of those 351 have been also quantified (Dame et al 2015).

1.3. NMR Spectroscopy

NMR is a physical phenomenon where certain atomic nuclei, when placed in a strong static magnetic field are perturbed by a weak oscillating magnetic field, respond by producing an electromagnetic signal at a frequency specific to the magnetic environment of the nucleus. This occurs near resonance, when the oscillation frequency matches the intrinsic frequency of the nuclei. The latter frequency depends on factors like the strength of the magnetic field, the chemical surroundings, and the magnetic properties of the isotope involved (Abraham, 1988).

NMR spectroscopy is widely used for its ability to determine the structure of organic molecules in solution, it is also an invaluable tool in studying molecular physics, crystals, and non-crystalline materials (Hore, 2015). It is inherently a quantitative technique, with signal intensity being directly proportional to the number of nuclei (e.g., ^{1}H, ^{13}C, ^{15}N, etc.) present in a sample. This characteristic makes NMR particularly well-suited for metabolomics, as the primary objective of the field is to quantify the concentration of each detectable metabolite within a biological sample (Crook & Powers, 2020).

In metabolomics, NMR is employed to analyse complex biological solutions such as biofluids, allowing researchers to identify and quantify the metabolites present. By generating high-resolution spectra, NMR provides a detailed snapshot of the metabolic state of a sample (Emwas et al, 2014). NMR-based metabolomics has proven particularly valuable in clinical applications, such as diagnosing metabolic disorders and exploring disease mechanisms. For instance, the method is used in newborn urine screening for inherited metabolic disorders (Embade et al., 2019) and has been applied to assess metabolic profiles in conditions like ischemic heart disease (Titma et al., 2019).

Moreover, NMR spectroscopy aids in tracking dynamic metabolic changes in biofluids over time, providing a robust framework for disease monitoring and biomarker discovery (Speyer and Baleja, 2021). Thus, NMR-based metabolomics is a crucial tool for enhancing our understanding of metabolic pathways and improving diagnostic capabilities in clinical settings (Wishart et al., 2022).

1H NMR spectra display as a series of peaks, each representing specific hydrogen atoms (protons) in a compound, reflecting their distinct chemical environments. The measuring process begins by placing the sample in a strong magnetic field, where the nuclei align. A radiofrequency pulse excites these nuclei, which then emit signals as they relax back to their original state. These emitted signals, initially captured as a time-domain Free Induction Decay (FID), are converted into a frequency-domain spectrum through Fourier transformation. The resulting spectrum shows peaks at chemical shifts (in ppm), which can be analysed to determine both the structure of the molecule and the concentration of specific compounds by integrating the area under each peak, as the area is directly proportional to the number of protons contributing to that signal (Speyer and Baleja 2022).

Chemical shifts cause variations in the NMR spectra, and these changes must be carefully considered when processing spectra, particularly for metabolites in a sample. It is important to account for factors such as baseline distortions, chemical shift referencing,

and potential overlaps between metabolite peaks. Properly calibrating the spectra to a standard reference, such as tetramethylsilane (TMS) or DSS, ensures accurate chemical shift measurements. Additionally, careful attention should be given to the resolution of peaks, as metabolites with similar chemical shifts may lead to spectral overlap, which can complicate the analysis and identification of individual metabolites (Saitô et al 2015).

2. Materials and Methods

2.1. Chemicals

The sodium phosphate buffer for serum samples was prepared using sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4), both obtained from Fisher Chemical. The potassium phosphate buffer for saliva samples was prepared using potassium dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate (K_2HPO_4), both sourced from Fisher Chemical. Milli-Q H_2O was used for high-purity water. The pH of the buffers was adjusted using potassium hydroxide (KOH) flakes, from Alfa Aesar, and hydrochloric acid (HCl) from Sigma Aldrich. Samples for NMR measurments contained 10% D_2O from the internal standard solution IS-2 (4.93 ± 0.05 mM DSS-D6, sodium 2,2-dimethyl-2-silapentane-5-sulfonate, 98 atom % D, with 0.1% w/v NaN_3 in D_2O) from Chenomx Inc. Imidazole from Sigma Aldrich was included to enable pH determination after NMR analysis.

2.2. Aparature

The following equipment and materials were used in the laboratory: calibrated Eppendorf micropipettes (100 µl, 200 µl, and 1000 µl) for precise liquid measurements, along with corresponding pipette tips. A pH meter (Mettler Toledo) was utilized for accurate pH measurements. Filtration was carried out using 3 kDa filters (Amicon). Samples were stored and processed in 1.5 ml and 2 ml Eppendorf tubes. For NMR analysis, 5 mm NMR tubes (Deutero) were employed. An Eppendorf centrifuge was used for sample centrifugation, while the analytical weighing balance was used for precise mass measurements. Additionally, the Microspin FV-2400 Minicentrifuge-Vortex machine was used for specific sample processing needs.

NMR spectra were measured at the NMR analysis laboratory at the University of Tartu using a Bruker Avance-III 700 MHz spectrometer (16.4 T) with prodigy TCI cryoprobe, for high-quality resolution to accurately analyse and resolve complex spectral data.

2.3. Ethical Aspects of Human Samples

Clinical data were collected at the North-Estonian Regional Hospital by Dr Marina Teras from patients with melanoma who were started on standard treatment. Patients and volunteers who were asked to participate in the study were informed both verbally and in writing, in coordination with the Tallinn Medical Research Ethics Committee (license no. 2855, issued to TUT Sirje Rüütel Boudinot 07/05/2019). Each participant signed the consent form that contained information about the objectives of the study, the design and the planned procedures. It also explained the risks involved and the measures to be taken to protect personal data. Personal data is kept in the medical records, according to official guidelines, and is accessible only to the medical doctor.

2.4. Sample Collection and Preparation

For the melanoma patients, WMS samples (4 ml) were collected by patients in the morning in fasted state, and blood samples (4 ml) were collected thereafter during the visit to Oncosurgery department of the North-Estonian Regional Hospital by the attending nurse. Samples were collected from three patients; three healthy control individuals were chosen from the volunteers by best matching age and sex (see Table 1). These volunteers also collected their own saliva samples in the morning in fasted state and blood samples were collected from them at Synlab. Samples were transported to TalTech at +4° C.

Table 1. Metadata of participants

ID	Age (years)	Sex	Weight (kg)	Height (m)	BMI	Stage
M1	61	F	80	1.7	28	IV
M2	72	M	88	1.82	27	IIIC
M3	75	M	60	1.72	20	IV
C1	66	F	64	1.72	22	Healthy
C2	55	M	109	1.88	31	Healthy
C3	73	M	67	1.66	24	Healthy

Blood samples were centrifuged to separate serum for 10 minutes at 1300 RCF at +4 °C, the serum was collected and frozen in aliquots of 500 µl. Saliva samples were centrifuged for 5 minutes at 3000 RCF at +4 °C to pellet cells and debris. The supernatant was collected and frozen in aliquots of 680 to 1000 µl.

Frozen samples of saliva were thawed on ice and centrifuged down at +4 °C. The supernatant was filtered with previously washed (5 times with 500 µl MQ water) 3 kDa Amicon filters by centrifugation at 12 000 RCF for 35 minutes at +4 °C. 540 µl of the filtrate was mixed with 60 µl of saliva NMR buffer (1.5 M KH_2PO_4/K_2HPO_4 buffer with 50 mM imidazole in IS-2, pH ~7.4) in 1.5 ml Eppendorf tube, vortexed for 2 minutes, and centrifuged at 14 000 RCF for 2 minutes at +4 °C. 550 µl of the supernantant was transferred into an 5 mm NMR tube and kept on ice until NMR measurements were taken.

Frozen samples of serum were thawed on ice and centrifuged down at +4 °C. The supernatant was filtered with previously washed (5 times with 500 µl MQ water) 3 kDa Amicon filters by centrifugation at 12 000 RCF for 35 minutes at +4 °C. 350 µl of the filtrate was mixed with 280 µl of serum NMR buffer (100 mM NaH_2PO_4/Na_2HPO_4 buffer with 50 mM imidazole, pH ~7.4) and 70 µl IS-2 in 1.5 ml Eppendorf tube, vortexed for 2 minutes, and centrifuged at 14 000 RCF for 1 minute at +4 °C. 600 µl of the supernantant was transferred into 5mm NMR tube and kept on ice until NMR measurements were taken.

2.5. NMR Measurements

The 1H spectra were acquired with TopSpin (Bruker) noesypr1d program at 4 °C, with the spectral width of 12 ppm, number of scans varied from 16 to 3200, for most samples 256. Solvent used for all samples was 90 % H_2O/10 % D_2O. Acquisition time was 4 s, mixing time 0.5 s, number of steady state scans 4.

2.6. Processing Spectra

The fid files acquired with TopSpin 3.2.7 were processed with the Processor module of the Chenomx NMR Suite v10 (Chenomx Inc.) software package. To ensure accuracy and consistency across all spectra, was developed detailed protocols for spectral processing. Phase correction was carried out using the automatic feature and fine tuned manually for most samples. For baseline correction Whittaker Spline with low equidistant breakpoint frequency and high smoothness was employed. Positions of breakpoints were corrected manually for all spectra. Shim correction and line broadening by 0.5 Hz were applied. The chemical shape indicator (CSI) was calibrated automatically. The pH of samples was calibrated manually by the imidazole peaks.

2.7. Quantification of Metabolites

Processed spectra were analysed with the Profiler module of the Chenomx NMR Suite v10. The first serum spectrum was manually profiled by matching the peaks to the Chenomx 700 MHz compound library, a database containing spectral signatures more than 250 metabolites. From the latter a custom serum library of compounds was created containing metabolites detected in the sample.

A compound was identified based on these criteria: at least one peak exceeded the noise level by at least a factor of 1.5 and the peak shape of that peak (and all others for compounds having several peaks) in the library matched or fitted in the pattern for overlapping peaks in the spectrum. Given the presence of many low-concentration metabolites, this threshold is crucial for avoiding false positives and ensuring that the detected signals represent actual metabolites rather than background noise (Lu et al 2017).

The other serum spectra were autofitted using the custom serum library, the fits were corrected manually. Additional compounds identified from the other spectra in the same manner as for the first spectrum were added to the serum library. Similar process was carried out for saliva spectra. The concentrations of metabolites resulting from peak fitting were calculated by the program based on the known concentration of the added internal reference compound, DSS.

2.8. Data Analysis

Metabolite concentration values were exported to Excel (Microsoft). Functions of the latter were used for data analysis. Before proceeding with calculations, the data was organized. Since the samples were diluted with the buffers, the concentration values exported from Chenomx Profiler were corrected corresponding to the dilution ratios (1:1 for serum, 9:1 for saliva to buffer) to obtain the concentrations of the metabolites in the biofluids. Principal component analysis (PCA) was conducted, and heat maps were acquired, with ClustVis, a web tool for visualizing clustering of multivariate data (BETA) - custom edition (Metsalu & Vilo, 2015).

3. Results

For all the 12 samples analysed spectra of sufficient quality were obtained for quantification of metabolites. 256 scans proved to be the optimal number for both serum and saliva spectra to acquire data for the detected compounds. Increasing the number of scans did not yield any additional metabolite signals. 64 scans were not enough to detect all the metabolites, the peaks belonging to many of the lower concentration compounds were not distinguishable from the noise. The selection of metabolites for comparative analysis was based on their detectability within samples: not all metabolites were included in the final analysis, as some were either absent or insufficiently represented across the samples.

3.1. Serum Metabolites

From the first serum sample analysed, of the first melanoma patient, designated M1, 53 metabolites were identified, 20 of which were difficult to quantify accurately, since peaks belonging to those metabolites were all either overlapping with others or near the noise level. The number of metabolites found altogether in the serum samples was 55.

Five metabolites (2-methylglutarate, 2-oxoisocaproate, 3-methyl-2-oxovalerate, erythritol, and inosine) were detected only once. Four metabolites (2-aminobutyrate, 2-hydroxyisovalerate, aspartate, and sarcosine) were found in two samples. The peak representing trimethylamine seemed to be present as well, but since it overlapped with what appeared to be an unidentifiable triplet, we were not able to quantify it, and it was omitted from further analysis.

45 metabolites were detected in at least half of the samples and therefore quantified (see Table 2). The concentrations for all these metabolites quantified in each serum sample analysed are given in Appendix 1.

Table 2. Mean values and ranges for metabolites found in at least half of the serum samples of melanoma patients (M) and healthy control individuals (C). All values are given in μM and are rounded to integer values. For the metabolites not detected in a sample, maximum possible values were given based on noise level at the locations of the peaks belonging to that metabolite.

Metabolite	M Mean (Range)	C Mean (Range)
3-Hydroxybutyrate	31 (28.4—35)	194 (44.6—490)
Acetate	33 (27—37.2)	60 (26.8—90)
Acetoacetate	14 (4.6—21.4)	42 (3—117.6)
Acetone	32 (24—38.8)	47 (33.6—54.4)
Alanine	342 (206.4—513.4)	333 (287.8—362.8)

Asparagine	50 (38.6—60.6)	51 (36.6—64.8)
Betaine	36 (25.4—42.2)	45 (28.4—70)
Carnitine	38 (35.4—39.6)	34 (12.8—45.2)
Choline	10 (6—14)	11 (10.6—11.4)
Citrate	121 (93.4—156.8)	139 (75.6—195.6)
Creatine	38 (16.2—56.6)	44 (16.8—78.4)
Creatinine	73 (60.6—88)	102 (73.2—158.6)
Dimethyl sulfone	15 (10.8—17.8)	20 (5.8—36.8)
Dimethylamine	2 (1.8—2.2)	4 (1.4—8)
Ethanol	404 (178.2—760.4)	201 (51—353.4)
Formate	31 (24—37.8)	27 (17—41.4)
Glucose	6613 (5215.8—8805.2)	5275 (3540.8—6185.8)
Glutamate	104 (94.8—110.6)	65 (49.8—78.4)
Glutamine	485 (282.2—607.4)	520 (351.4—646.6)
Glycine	318 (145.4—526)	393 (248.8—484.4)
Histidine	68 (35—88.8)	80 (75—82.4)
Hypoxanthine	26 (13.2—38.4)	9 (7.4—9.6)
Isoleucine	77 (40.6—99.8)	59 (49.2—67.6)
Lactate	3265 (1569.8—4460.2)	2426 (2050.6—2729.4)
Leucine	135 (84.4—161.4)	102 (90.4—121.4)
Lysine	165 (109.8—203)	158 (127—187.8)
Mannose	95 (55—160.6)	50 (31.4—65.6)
Methanol	76 (57.4—94.8)	207 (57.8—482.6)
Methionine	28 (15.8—37.8)	28 (20.6—38.4)
N,N-Dimethylglycine	4 (2.8—6)	3 (2.4—3.8)
O-Acetylcarnitine	6 (4.6—9.2)	11 (4.6—20.8)
Ornithine	52 (33.4—63.8)	59 (48.8—64.4)
Phenylalanine	58 (44.2—66)	50 (43.4—57)
Proline	199 (114.4—275)	226 (184—268.6)
Pyroglutamate	19 (17.2—24)	42 (24.2—70.2)
Pyruvate	80 (39.2—110)	48 (43—55)
Serine	84 (44.8—136)	136 (98.2—193.8)
Succinate	5 (3.4—6.8)	4 (1.8—4.8)
Taurine	69 (51.8—77.8)	75 (65.8—82.2)
Threonine	103 (74.4—132.4)	143 (97.6—168.4)
Tyrosine	85 (49.2—102.8)	73 (47.8—85.8)
Urea	2563 (2317—3001.8)	2191 (820.6—3016)
Valine	245 (135—335.6)	216 (185.4—232.8)
myo-Inositol	16 (0—29.6)	18 (5.4 – 54)

Principal component analysis (PCA) revealed that one of the control subjects is clustering with patients, see Figure 1 in Appendix 3.

3.2. Saliva Metabolites

From the first saliva sample analysed, of the first melanoma patient (M1) 58 metabolites were identified, 17 of which were difficult to quantify accurately. 63 metabolites altogether were detected in the saliva samples. 10 metabolites (1,3–dihydroxyacetone, 2–aminobutyrate, 2–hydroxyisobutyrate, acetone, aspartate, cadaverine, glucose, O–phosphocholine, threonine, and uracil) were found only on one occasion. 9 metabolites (β–alanine, carnitine, erythritol, fumarate, hydroxyacetone, hypoxanthine, methionine, tryptophan, and xanthine) were detected in two samples. Creatinine peaks seemed to be present at least in one sample, but both overlapped with other peaks, one in a region having multiple unidentified peaks so it was omitted from further analysis. The presence of ornithine and lysine cannot be ruled out; however, all their cluster are found in overlapping regions.

47 metabolites were detected in at least half of the samples and therefore quantified (see Table 3). The concentrations are given in Appendix 2.

Table 3. Mean values and ranges for metabolites found in at least half of the saliva samples of melanoma patients (M) and healthy control individuals (C). All values are given in μM and are rounded to integer values. For the metabolites not detected in a sample, maximum possible values were given based on noise level at the locations of the peaks belonging to that metabolite.

Metabolite	M mean (range)	C mean (range)
2-Hydroxyisovalerate	3 (2 — 4)	4 (3 — 4)
3-Phenylpropionate	34 (4 — 83)	18 (7 — 37)
4-Hydroxyphenylacetate	48 (20 — 96)	31 (5 — 67)
5-Aminopentanoate	857 (804 — 945)	387 (10 — 811)
Acetate	13491 (7797 — 19331)	8346 (5162 — 10211)
Acetoin	32 (17 — 40)	103 (4 — 293)
Alanine	249 (31 — 640)	40 (13 — 71)
Arginine	57 (49 — 65)	13 (5 — 18)

Butyrate	499 (377 — 601)	134 (13 — 317)
Choline	47 (9 — 119)	19 (4 — 47)
Creatine	22 (9 — 37)	12 (6 — 22)
Desaminotyrosine	44 (13 — 99)	17 (0 — 36)
Dimethyl sulfone	20 (12 — 33)	21 (6 — 38)
Dimethylamine	5 (4 — 5)	4 (3 — 5)
Ethanol	8527 (145 — 18111)	247 (186 — 320)
Ethanolamine	144 (61 — 275)	75 (5 — 210)
Formate	324 (21 — 834)	189 (26 — 373)
Fucose	244 (29 — 505)	32 (17 — 61)
Galactose	112 (66 — 153)	35 (19 — 50)
Glycine	692 (608 — 770)	231 (161 — 270)
Histidine	66 (25 — 114)	17 (5 — 35)
Isobutyrate	133 (34 — 306)	72 (1 — 115)
Isocaproate	23 (10 — 46)	21 (2 — 51)
Isoleucine	87 (15 — 228)	16 (13 — 18)
Isovalerate	44 (17 — 98)	36 (1 — 58)
Lactate	1826 (70 — 4877)	535 (4 — 1593)
Leucine	208 (32 — 555)	34 (32 — 36)
Methanol	114 (51 — 171)	125 (65 — 235)
Methylamine	15 (5 — 20)	22 (5 — 40)
Phenol	19 (0 — 41)	27 (15 — 40)
Phenylacetate	49 (25 — 73)	42 (7 — 78)
Phenylalanine	120 (29 — 268)	30 (28 — 35)

Proline	734 (568 — 933)	73 (20 — 122)
Propionate	3489 (2133 — 5750)	1927 (901 — 2586)
Putrescine	492 (180 — 909)	101 (29 — 176)
Pyruvate	151 (64 — 207)	62 (1 — 180)
Sarcosine	38 (9 — 89)	11 (4 — 18)
Succinate	971 (31 — 2769)	13 (3 — 29)
Taurine	508 (15 — 1305)	162 (121 — 240)
Trimethylamine	2 (0 — 6)	1 (1 — 2)
Tyrosine	153 (52 — 313)	38 (28 — 54)
Urea	348 (211 — 544)	5 (0 — 16)
Valine	180 (10 — 488)	46 (26 — 63)

In the PCA the clusters of melanoma patients and those of the healthy controls can be seen as nearly separate, see Figure 2 in Appendix 3. Combining the values from both the serum and the saliva analysis yielded a similar PCA result as the serum analysis alone, see Figure 3 in Appendix 3.

Discussion

This study aimed to profile metabolite concentrations in saliva and serum samples from melanoma patients and healthy controls, using NMR–based metabolomics. The primary goal was to generate a detailed concentration profile of targeted metabolites and assess their potential relevance in distinguishing between the two groups. While the study revealed notable differences in metabolite concentrations, the limited sample size constrained statistical power and precluded robust conclusions regarding broader biological or mechanistic interpretations.

The metabolite profiling approach enabled the detection and quantification of several metabolites across saliva and serum samples. In saliva, metabolites such as acetate, ethanol, alanine, and propionate exhibited higher concentrations in melanoma patients than in controls. For instance, ethanol concentration in melanoma patients was strikingly elevated compared to controls, suggesting potential relevance as a biomarker.

In serum, metabolites like alanine, glucose, and lactate were found at elevated concentrations in melanoma patients, consistent with known metabolic adaptations in cancer, such as increased glycolysis and amino acid metabolism. Elevated levels of branched–chain amino acids (leucine, isoleucine, and valine) were also observed, indicative of their heightened demand in tumour metabolism.

While these findings align with known cancer–associated metabolic changes, the study's primary objective was limited to metabolite concentration profiling. Establishing robust statistical associations or causal links requires larger sample sizes and additional data.

Several challenges emerged during this study that impacted the robustness of the findings. One significant limitation was the small sample size, which restricted the statistical power and made it difficult to draw definitive conclusions about the significance of the observed differences in metabolite concentrations. Additionally, confounding factors such as diet, medication, and comorbid conditions could have influenced metabolite levels, potentially introducing bias into the profiles and complicating the interpretation of the results. Lastly, the complexity of NMR spectra, including the presence of overlapping peaks—especially for sugars and low–concentration metabolites (see Appendix 4)—posed challenges in metabolite identification and quantification, which may have affected the accuracy of the results.

This study successfully profiled metabolite concentrations in saliva and serum samples from melanoma patients and healthy controls, revealing several differences that may warrant further investigation. While the limited sample size precluded statistical validation or mechanistic interpretation, the findings highlight the potential of metabolomics in characterizing disease–associated metabolic changes. Future studies with larger cohorts and integrated analyses are essential to build on these preliminary insights and translate them into clinically actionable tools.

Conclusions

This investigation conducted on a very limited set of samples shows that notable differences can be found in salivary metabolomic profiles of melanoma patients compared to healthy individuals.

Although the study had limitations due to time constraints, laying this groundwork was critical for future efforts to detect key metabolites and monitor their concentration fluctuations under specific conditions.

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Abstract

Metabolomic profiles of serum and saliva from melanoma patients and healthy control subjects were ascertained and compared in this study to pave the way for developing early detection and treatment monitoring methods. Altogether six NMR spectra were obtained and profiled.

The metabolite profiling approach enabled the detection and quantification of several dozens of metabolites across saliva and serum samples. In saliva, metabolites such as acetate, ethanol, alanine, and propionate exhibited higher concentrations in melanoma patients than in controls. For instance, ethanol concentration in melanoma patients was strikingly elevated compared to controls, suggesting potential relevance as a biomarker.

In serum, metabolites like alanine, glucose, and lactate were found at elevated concentrations in IIIC–IV stage melanoma patients, consistent with known metabolic adaptations in cancer. Elevated levels of branched–chain amino acids (leucine, isoleucine, and valine) were also observed, indicative of their heightened demand in tumour metabolism.

Kokkuvõte

Selles uurimistöös tuvastati melanoomiga patsientide ja tervete kontrollisikute seerumi ja sülje metaboolsed profiilid, sillutamaks teed tulevaste varajase diagnostika ja ravi jälgimise meetodite arendamiseks. Selleks mõõdeti ja profileeriti tuumamagnetresonantsspektroskoopiaga kuus proovi.

Kehavedelike proovidest leiti kümneid erinevaid metaboliite, millest enamiku puhul määrasti kindlaks nende kontsentratsioonid. Süljes esinesid metaboliidid, nagu atsetaat, etanool, alaniin ja propionaat, IIIC–IV staadiumi melanoomiga patsientidel kõrgemates kontsentratsioonides kui kontrollrühmas. Näiteks oli etanooli kontsentratsioon melanoomiga patsientidel võrreldes kontrollidega märkimisväärselt kõrgem, mis viitab sellele, et tegemist võib olla olulise biomarkeriga.

Melanoomiga patsientidel leiti seerumis metaboliite, nagu alaniin, glükoos ja laktaat, kõrgendatud kontsentratsioonides, mis on kooskõlas teadaolevate metaboolsete muutustega vähi korral, nagu suurenenud glükolüüs ja aminohapete metabolism. Hargnenud ahelaga aminohapete (leutsiin, isoleutsiin ja valiin) määr melanoomi grupis oli samuti kõrgem, mis viitab nende suurenenud nõudlusele kasvaja metabolismis.

Appendices

Appendix 1. Concentrations of Serum Metabolites, in Samples Measured (µM)

	M1	M2	M3	C1	C2	C3
3-Hydroxybutyrate	15.1 ±1.2	14.2 ±1.1	17.5 ±0.6	22.3 ±0.3	23.2 ±0.4	245 ±1
Acetate	18.6 ±0.5	13.5 ±2.3	17.1 ±0.5	13.4 ±1.5	45 ±0.5	31.1 ±2
Acetoacetate	2.3 ±0.4	10.7 ±0.4	8.2 ±0.6	1.5 ±0.5	2.2 ±0.4	58.8 ±0.8
Acetone	16.7 ±0.3	19.4 ±0.2	12 ±0.2	16.8 ±0.1	25.9 ±0.1	27.2 ±0.3
Alanine	256.7 ±2.1	152.5 ±0.5	103.2 ±0.5	181.4 ±0.6	143.9 ±0.6	174.9 ±1
Asparagine	30.3 ±0.5	19.3 ±0.7	25.1 ±3	25.8 ±0.4	18.3 ±1	32.4 ±0.8
Betaine	12.7 ±2.2	21.1 ±0.5	20 ±1.3	14.2 ±1.3	18.4 ±1.5	35 ±0.3
Carnitine	17.7 ±1.8	19 ±1	19.8 ±0.8	21.7 ±0.7	6.4 ±1.2	22.6 ±0.3
Choline	7 ±0.1	5.6 ±0.2	3 ±0.1	5.3 ±0.2	5.7 ±0.1	5.5 ±0.2
Citrate	78.4 ±1.2	56.8 ±1.2	46.7 ±1	72.2 ±1.2	37.8 ±0.8	97.8 ±2.1
Creatine	28.3 ±0.8	8.1 ±0.2	20.9 ±0.5	39.2 ±0.7	18.3 ±0.5	8.4 ±0.7
Creatinine	44 ±0.7	35.5 ±0.2	30.3 ±0.3	36.6 ±0.4	37.3 ±0.5	79.3 ±1
Dimethyl sulfone	7.6 ±0.2	5.4 ±0.2	8.9 ±0.1	8.5 ±0.2	2.9 ±0.2	18.4 ±0.5
Dimethylamine	1 ±0.1	0.9 ±0	1.1 ±0.1	0.7 ±0	4 ±0.2	1.3 ±0.1
Ethanol	137.3 ±1.5	89.1 ±1.6	380.2 ±2.2	99.4 ±0.4	176.7 ±1.2	25.5 ±1.6
Formate	12 ±0.4	14.9 ±0.2	18.9 ±0.8	8.5 ±0.2	20.7 ±0.3	10.7 ±0.6
Glucose	2607.9 ±19.9	2909.2 ±13.1	4402.6 ±12.7	3048.9 ±8.8	1770.4 ±11.3	3092.9 ±10.7
Glutamate	53.1 ±1.7	47.4 ±4.4	55.3 ±1.1	24.9 ±0.7	33 ±1	39.2 ±1
Glutamine	282.5 ±2.7	303.7 ±2.6	141.1 ±1.5	281.4 ±1.6	175.7 ±2.1	323.3 ±2.9
Glycine	263 ±8	140.7 ±3.5	72.7 ±0.9	223 ±1.9	124.4 ±2.8	242.2 ±2.2
Histidine	40.5 ±0.4	44.4 ±0.3	17.5 ±0.5	40.9 ±0.6	37.5 ±0.5	41.2 ±0.5
Hypoxanthine	19.2 ±0.3	6.6 ±0.3	13.8 ±0.8	4.8 ±0.3	3.7 ±0.7	4.5 ±0.3
Isoleucine	44.9 ±0.3	49.9 ±0.7	20.3 ±0.5	30.4 ±0.2	24.6 ±0.2	33.8 ±0.3
Lactate	1883.1 ±19.8	784.9 ±5.6	2230.1 ±6.9	1364.7 ±8	1249.4 ±3.8	1025.3 ±8.1
Leucine	79.2 ±1.7	80.7 ±1.9	42.2 ±0.7	60.7 ±0.8	47.6 ±1.2	45.2 ±3.2
Lysine	91.6 ±1.1	101.5 ±0.6	54.9 ±1	80.2 ±0.8	63.5 ±0.9	93.9 ±2.2
Mannose	34.4 ±0.3	27.5 ±0.5	80.3 ±3.4	26.4 ±0.5	15.7 ±1.2	32.8 ±1
Methanol	47.4 ±3	38 ±4.5	28.7 ±3.8	40.2 ±2.6	241.3 ±4.1	28.9 ±6.6

Methionine	18.9 ±0.8	15.9 ±0.4	7.9 ±0.5	12.6 ±0.6	10.3 ±0.5	19.2 ±0.8
N,N-Dimethylglycine	1.4 ±0	3 ±0.1	2.3 ±0.3	1.3 ±0.1	1.2 ±0.1	1.9 ±0.1
O-Acetylcarnitine	4.6 ±0.1	2.3 ±0.2	2.4 ±0.1	3.9 ±0.2	2.3 ±0.1	10.4 ±0.2
Ornithine	30.1 ±0.9	31.9 ±0.7	16.7 ±0.7	32.2 ±0.9	31.3 ±0.8	24.4 ±2.3
Phenylalanine	33 ±0.1	22.1 ±0.4	32.3 ±0.4	25.1 ±0.2	21.7 ±0.2	28.5 ±0.6
Proline	137.5 ±2.7	103.7 ±5.6	57.2 ±1.2	112.6 ±1.1	92 ±1.4	134.3 ±1.5
Pyroglutamate	12 ±0.7	8.6 ±0.7	8.6 ±1.2	12.1 ±0.7	35.1 ±1.4	15.3 ±0.9
Pyruvate	44.9 ±0.7	19.6 ±0.7	55 ±0.3	22.3 ±0.3	27.5 ±0.3	21.5 ±0.2
Serine	68 ±2.2	36.1 ±3.3	22.4 ±2.4	96.9 ±4	49.1 ±5.5	57.7 ±7.7
Succinate	3.4 ±0.2	2.3 ±0.1	1.7 ±0.2	2.4 ±0.1	0.9 ±0.2	2.4 ±0.1
Taurine	25.9 ±12	38.9 ±3.3	38.4 ±7.1	38.2 ±4.7	32.9 ±6.5	41.1 ±3.9
Threonine	66.2 ±0.5	51.8 ±1.1	37.2 ±2.9	80.8 ±0.4	48.8 ±1.5	84.2 ±7.8
Tyrosine	51.4 ±0.3	51.2 ±0.3	24.6 ±0.4	42.9 ±0.6	23.9 ±0.5	42.5 ±0.6
Urea	1184.9 ±1.5	1500.9 ±3.1	1158.5 ±7.7	1508 ±2	410.3 ±6.7	1368.9 ±3.5
Valine	132.5 ±1	167.8 ±1.9	67.5 ±0.5	114.7 ±0.4	92.7 ±0.2	116.4 ±0.6
myo-Inositol	14.8 ±1.5	0 ±0	9.7 ±0.5	16.2±1.4	8.2±0.1	23.3±0.5

Appendix 2. Concentrations of Saliva Metabolites in Samples Measured (μM)

Metabolite	M1	M2	M3	C1	C2	C3
Acetate	12009.4 ±28.2	7017.5 ±26.8	16528.4 ±55.9	9285.8 ±34.3	4333.7 ±22.4	8645.2 ±30.5
Alanine	30.6 ±6.8	28.2 ±3.6	576.2 ±16.7	9.1 ±1.8	67.2 ±0.7	36.5 ±1.9
Choline	5.6 ±0.5	11.2 ±1.3	97 ±1.1	5.3 ±0.1	38.7 ±0.2	4.2 ±0.1
Ethanol	130.7 ±2.2	6593.8 ±12.6	16299.7 ±71.3	289.3 ±1.7	211.8 ±1	167.5 ±1.3
Formate	104.5 ±0.6	750.6 ±3.8	18.5 ±0.6	22 ±0.6	335.6 ±3.4	151.9 ±0.7
Methanol	45.7 ±3.1	108.4 ±0.6	154.2 ±2	12.6 ±10.5	211.3 ±1.5	58.5 ±0.5
Phenylalanine	56.7 ±0.2	26 ±2.1	241.2 ±0.8	27.9 ±1.1	25.3 ±1.9	31.3 ±1
Propionate	2325.7 ±7.7	1920 ±5.4	5175.4 ±28.9	2501 ±10.4	811.1 ±2	2063 ±6
Putrescine	349 ±9	161.6 ±2.7	818 ±14	205.5 ±2.8	26.3 ±0.7	87.3 ±1.6
Taurine	128.5 ±6.9	166.8 ±1.8	1174.9 ±15.2	124.6 ±2.7	108.9 ±1.8	216.2 ±1.2
Tyrosine	83.4 ±2.4	44.6 ±0.8	282 ±4.3	48.4 ±0.5	35.8 ±0.9	28.7 ±0.7
Valine	39.3 ±0.7	9 ±1.5	438.9 ±8.5	45.2 ±0.4	23.8 ±0.8	56.3 ±1.4
Fucose	177.9 ±2.9	26.1 ±2.9	558.8 ±4.8	55.2 ±1.2	16.3 ±0.8	14.9 ±1.8
Glycine	693.3 ±3.7	546.9 ±3	628.1 ±19.9	281.4 ±7.7	144.5 ±1.4	242.8 ±2.1
Isobutyrate	52.8 ±3.9	30.8 ±2	149.1 ±11.2	165.9 ±6.2	0.8 ±0.8	89.7 ±3.4
Isoleucine	13.1 ±0.6	15.5 ±1.3	205.2 ±7.4	16.9 ±0.5	12 ±0.3	15.9 ±1.3
Methylamine	18 ±0.1	4.2 ±0.3	17.4 ±3.5	37 ±0.2	4.4 ±0.5	18.3 ±0.2
Butyrate	432.2 ±5.7	348.6 ±5.4	562.4 ±30.6	74.4 ±1.3	16.6 ±1.2	31 ±1.2
5-Aminopentanoate	590.6 ±18.1	723.3 ±3.1	607.9 ±13.7	757 ±2.8	7.7 ±0.4	302.2 ±2.1
Isovalerate	16.1 ±4.2	14.9 ±2.3	88.3 ±11.5	53.1 ±4.6	0.8 ±1	43.9 ±2.8
Dimethylamine	4.3 ±0.2	4 ±0.5	3.9 ±2.2	4.2 ±0.1	2.6 ±0	4.8 ±0

Leucine	32.2 ±2.6	29 ±3.9	499.1 ±20.7	16 ±1.1	28.3 ±1.1	30 ±3.4
Proline	629.3 ±15.5	511.6 ±2.1	839.6 ±6.8	79.1 ±4	68.1 ±5.7	18.1 ±1
Sarcosine	14.3 ±0.9	7.8 ±1.4	79.7 ±7.6	3.7 ±1.2	3.9 ±0.2	15.8 ±0.3
Lactate	478.6 ±1.9	63 ±0.9	4389 ±27.9	3.1 ±1.2	1433.5 ±8.7	8.8 ±0.9
Pyruvate	143.3 ±8.1	57.5 ±15.2	186.2 ±12.7	2.5 ±1.5	161.6 ±1	1.3 ±0.6
Desaminotyrosine	15.8 ±1.4	12 ±3.5	88.7 ±7.3	33.2 ±0.8	1.1 ±0.5	14.4 ±1.4
Isocaproate	8.5 ±1.7	9.4 ±2.7	41.5 ±9.1	47.6 ±2.9	2.1 ±0.8	7.1 ±3.4
Dimethyl sulfone	9.2 ±0.8	10.6 ±0.2	29.9 ±3.7	15.6 ±0.2	5 ±0.2	34.6 ±0.2
Phenylacetate	41 ±1.5	22.8 ±1.8	65.9 ±4.2	77.5 ±1.2	6.4 ±1.4	36.4 ±1
3-Phenylpropionate	12.7 ±1.2	3.9 ±0.7	66.4 ±3.7	29.5 ±1	0.9 ±0.4	8.8 ±0.6
Ethanolamine	85.1 ±3.9	54.6 ±1	247.9 ±22.4	8.7 ±1.4	189.2 ±1.8	4.5 ±0.8
Phenol	37.2 ±1.2	10.9 ±2	14.3 ±0.7	39.5 ±0.4	13.3 ±1.4	23.6 ±0.6
Creatine	32.8 ±0.9	8.5 ±1.2	35.4 ±7	2.9 ±1.4	27.2 ±1.2	6 ±0.6
Succinate	100.9 ±3.1	28 ±7.5	2492.4 ±6.9	2.5 ±0.9	26.3 ±0.3	4.8 ±0.1
Acetoin	36.2 ±2.3	33.7 ±1.3	35.6 ±6.1	7.6 ±1.4	263.7 ±1.2	5.7 ±0.8
2-Hydroxyisovalerate	3 ±0.7	1.5 ±1.1	4 ±3.5	4 ±0.3	1.4 ±0.5	5.1 ±0.1
Urea	189.8 ±2.4	490.8 ±6.5	259.1 ±3.9	15 ±2.4	14.4 ±2.1	15.9 ±2.3
4-Hydroxyphenylacetate	58.6 ±2.8	18.1 ±0.8	15.7 ±2	68.2 ±2.4	3.8 ±0.5	13.7 ±0.6
Histidine	53.7 ±2.6	22.1 ±1.1	102.8 ±2.6	9.7 ±0.8	31.4 ±0.4	4.3 ±0.5
Galactose	195.5 ±3.5	59.5 ±3.2	104.6 ±2.2	13.3 ±1.9	45.4 ±1.2	31.7 ±2.4
Isopropanol	1.8 ±0.5	21.7 ±4.6	133.1 ±24.9	1.2 ±0.4	1 ±0.4	2.9 ±0.3
Arginine	63.1 ±4.8	10.3 ±5.6	57.3 ±5.2	2.6 ±0.6	10.2 ±1	12.3 ±1.7
Aspartate	20 ±1.6	7.5 ±2.6	81.4 ±8.7	6.4 ±0.7	12.6 ±0.4	9.2 ±1
Glucose	8.1 ±5.9	28 ±3.4	1180 ±14.4	8.8 ±1.4	66.9 ±2.5	10.5 ±1.7

Methionine	1.1 ±0.6	4.6 ±0.7	76.5 ±6.3	6.2 ±0.4	5.8 ±0.5	1.8 ±0.5

Appendix 3. Principal Component Analysis of Serum and Saliva Samples

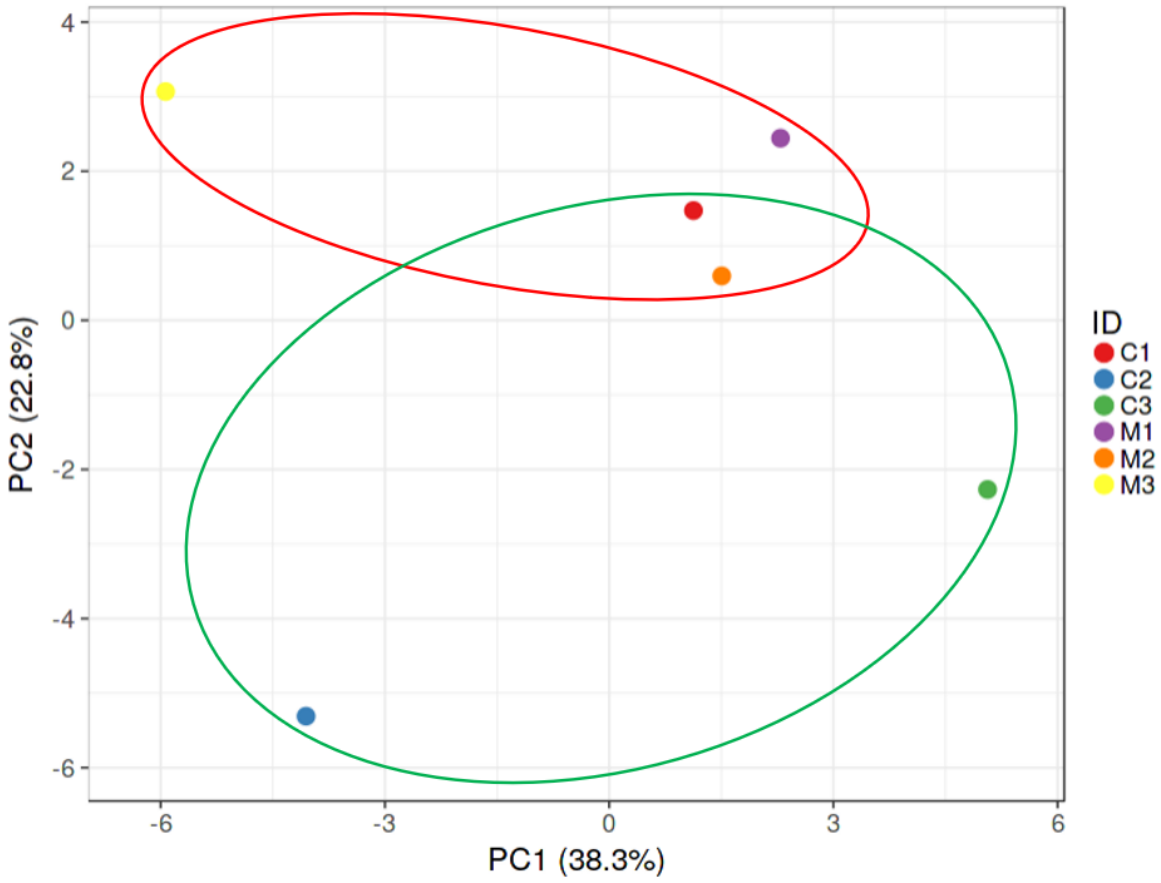

Figure 1. PCA results of the quantified serum metabolites. The red ellipse encompasses melanoma samples and the green ellipse the controls.

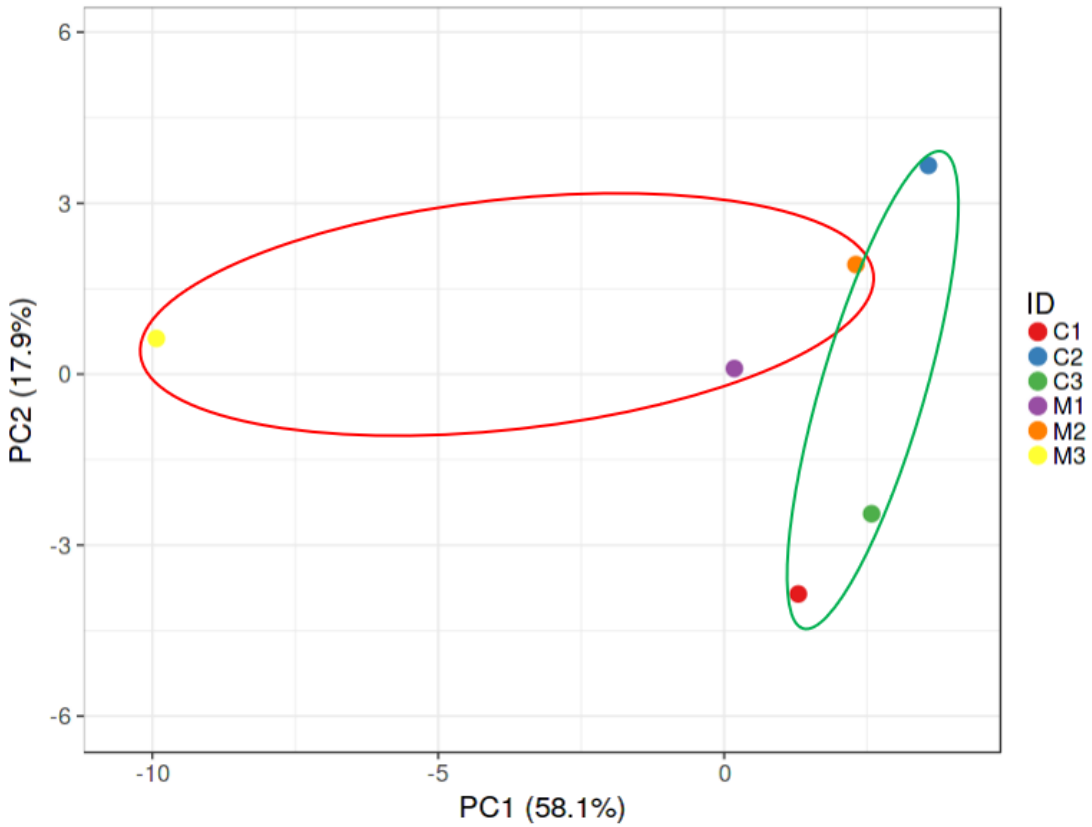

Figure 2. PCA results of the quantified saliva metabolites. The red ellipse encompasses melanoma samples and the green ellipse the controls.

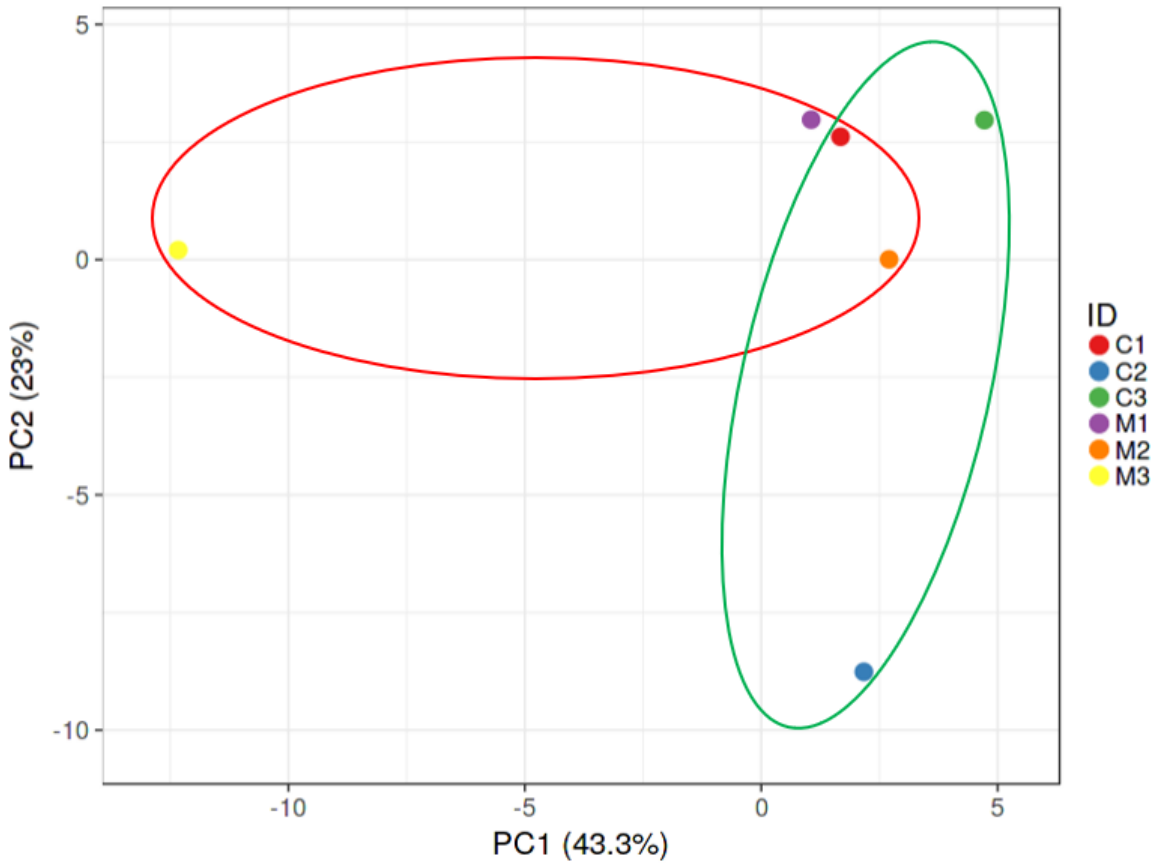

Figure 3. PCA of the quantified serum and saliva metabolites. The red ellipse encompasses melanoma samples and the green ellipse the controls.

Appendix 4. Overlaying Metabolites in a Serum Spectrum

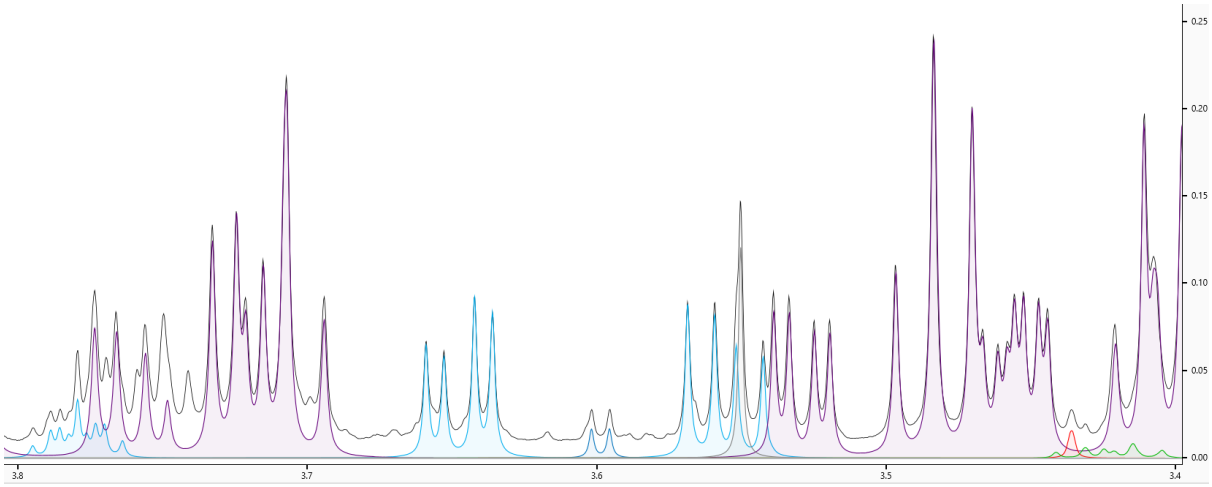

Figure 4. A segment of an annotated 700 MHz1H NMR spectrum of serum from a healthy control subject (C3). The black line represents the spectrum line, spectrum, various coloured lines and areas highlight the distinct clusters at chemical shifts corresponding to different metabolites: purple represents glucose, blue corresponds to glycerol, red indicates proline, and green marks acetoacetate.