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Salivary and Urine Metabomic Profile of Patients Living with HIV/AIDS, and Its Association with Periodontal Disease

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Abstract: The metabolome is the composition of all molecules present in an organism, metabolomics science allows us to qualitatively and quantitatively analyze metabolites and understand the biochemical state in response to genetic and environmental changes. The purpose of the study was to identify the metabolomic profile of biofluids (saliva and urine) of patients living with HIV/AIDS (PLHIV/AIDS),and establish a possible association of metabolites with periodontal disease (PD). It was an analytical and descriptive study composed of 65 patients seen at the Center for Studies and Care for Special Patients (CEAPE-UNIP), who were divided into 4 predetermined groups (G), G1-PLHIV/AIDS and without PD, G2- PLHIV/Aids and with PD, G3-people without HIV/Aids and without PD, G4-people without HIV/Aids and with PD. The results showed that in relation to urine, G2 has the highest mean area of concentration of metabolites. There are statistically significant differences between G1 and G2. The area of G2 is greater than G3, and greater than G4. There are no differences between G3 and G4, neither between G1 and G3, nor between G1 and G4. Regarding saliva, there are no statistically significant differences when comparing the groups for the mean areas of concentration of metabolites. We conclude that it was possible to distinguish the metabolic profile of PLHIV/Aids with periodontal disease and without periodontal disease, some metabolites are over expressed in PLHIV/Aids and with PD. Most of the metabolites of the carbohydrate group are under expressed in PLHIV/AIDS, even in systemic compensation situations these patients presented a different metabolic profile.

Keywords: metabolomics; biofluids; metabolites; biomarkers; HIV

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1. Introduction

Acquired immunodeficiency syndrome (AIDS) was identified in 1981, its publication in the United States occurred between 1982 and 1983, in this period the human immunodeficiency virus (HIV) was identified. In the beginning it was considered fully associated with homosexuality and prostitution, suffering discrimination and prejudice by the society of the time. AIDS has been associated with another pathology called pneumonia by Pneumocystis, it is a defining disease of AIDS in HIV-infected patients, presents itself as the most common lung disease in this population. HIV transmission occurs mainly through sexual intercourse, also by inoculation of contaminated blood, needles and syringes and vertical transmission (from mother to child). As the HIV virus affects the immune system, the first symptoms and signs of the disease appear, in a more advanced stage, it may progress to progressive and severe consumptive syndrome, with possible evolution to death.[1–4]

In the ranking of non-communicable diseases, those that affect the oral cavity occupy the first place, according to the World Health Organization (WHO), recent research has shown the relationship of periodontitis with other systemic conditions, including coronary disease, rheumatoid arthritis, Alzheimer's, some types of cancer, respiratory ailments, erectile dysfunction, among others. [5]

A detailed history to obtain information about any comorbidity presented by the individual, previous treatments and medications used, is extremely important, in addition, it is essential to know the Dental Surgeon about the disease, its characteristics and consequences, especially in the oral cavity. Several opportunistic diseases are associated with HIV/AIDS, and in relation to oral diseases, there are a number of bacterial, fungal and viral manifestations, in addition to possible malignancies. It is noteworthy that periodontal disease can appear abruptly, depending on the current profile of the individual's immunity. HIV infection predisposes patients to certain oral health problems, HIV can have a direct action on the pathogenicity of periodontal disease, with or without the development of AIDS. [6–9]

The metabolome is the composition of all molecules present in an organism, the science of metabolomics allows us to qualitatively and quantitatively analyze the possible metabolites present in cells, organs and organisms and to understand the biochemical state of an organism in response to genetic and environmental changes. Given the evolution of techniques and knowledge about the mechanisms of some pathologies, metabolomics has been used as an effective tool in the diagnosis and identification of metabolites secreted by microorganisms present in the oral cavity. This tool has the role of monitoring the amount of metabolites such as amino acids, lipids and sugars with the exception of DNA, RNA and proteins, being possible to understand the dynamics of diseases in the body. The clinical use of the metabolome for the investigation of saliva and urine is related to the low cost, the possibility of processing the samples and the ease of obtaining this material, since it is a less invasive form when compared to the collection of other biological specimens. [8,10–13] Concerning the relevance of the study, it can be justified by the fact that no research in the field of metabolomics concerning the relationship between PD and HIV was found.

Through the analytical techniques commonly used in metabolomics, which are liquid or gas chromatography associated or not with mass spectrometry and infrared spectroscopy, it is feasible to study the possible associations of periodontal disease with other systemic conditions, therefore, the objective of the present The aim of this study was to identify the metabolomic profile of biofluids (saliva and urine) of patients living with HIV/AIDS, and to verify a possible association with periodontal disease.

2. METHODS AND MATERIALS

A total of 118 patients were evaluated, 62 with HIV/AIDS treated at CEAPE (Center for the Study and Care of Special Patients) and 56 patients at the Integrated Clinic of the Dentistry Course at the Universidade Paulista de São Paulo (UNIP - SP). included in the research, with 32 patients diagnosed with HIV/AIDS (seropositive) and 33 without a diagnosis of HIV/AIDS (seronegative), both attended at the CEAPE clinics and the Integrated Clinic of the Dentistry course at the Universidade Paulista

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de São Paulo (FOUNIP – SP).The study was conducted in such a way that patients were clinically allocated to pre-selected groups, randomly distributed within the respective groups, as follows:

Group 1: patients living with HIV/AIDS and without periodontal disease (n=13).

Group 2: patients living with HIV/AIDS and periodontal disease (n=19).

Group 3: individuals without diagnosis for HIV/AIDS seropositivity and without periodontal disease (n=17).

Group 4: individuals without HIV/AIDS seropositivity diagnosis and with periodontal disease (n=16).

Experimental unit: Patients

Sample size: 65 subjects – Allocated into 4 groups (**G1, G2, G3, G4**).

This study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee in Research with Human Beings of Universidade Paulista – UNIP, with the number of opinion 4.613.288. Patients were informed about the objectives and conditions of the study, and those who agreed to participate signed the Informed Consent Form (ICF).

Data were collected regarding gender, age, skin color, CD4, viral load, habits, comorbidities, oral diseases, use of medications and time of diagnosis of HIV/AIDS pathology (G1 and G2). Periodontal examination was performed by two examiners (VBCS and ESS), after calibration, and the Kappa calibration coefficient inter and intra examiner were calculated, following all biosafety precepts.

Inclusion criteria: patients living with HIV/AIDS confirmed by laboratory tests (G1 without periodontitis and G2 with periodontitis) and patients without confirmed seropositivity for HIV/AIDS (G3 without periodontitis and G4 with periodontitis), with greater than or equal to 3 mm of clinical probing depth (PS), with bleeding and using oral immunosuppressants prescribed by the physician. Comorbidities were not excluded.

Exclusion criteria: periodontal treatment in the last 6 months (G2 and G4), pregnant women, nursing mothers, patients who had cough, edema in the lower limbs, fever, dyspnea, pulse therapy with prednisone (3 to 5 days), systemic arterial hypertension (SAH) uncontrolled and completely edentulous.

Methodology

Clinical probing depth (PS)

Performed with relative isolation, using a millimeter probe from 1 to 10 mm and artificial lighting. The probe was introduced into the marginal gum region and inserted into each tooth, and the measurement was noted by sextant in the clinical record (Periogram - UNIP standard record).

A measurement ≥ 3mm was considered in relation to the probing depth, and when there was bleeding it was classified as the presence of periodontal disease. The criteria also followed the new classification of the American Periodontics Association in relation to the Parameter - Clinical Insertion Level (NIC):

NIC 1-2 mm = incipient - Stage l;

IAS 3-4 mm = moderate – Stage ll;

NIC ≥ 5 mm severe or very severe - Stage lll or lV.

Examiner calibration

The examiners responsible for the measurements were calibrated prior to the initial examination (intra-examiner Kappa = 0.89 and inter-examiner Kappa = 0.85, respectively). Three patients with periodontitis were evaluated in order to collect PS data, with a 24-hour interval between measurements.

Collection and storage of biological specimens (saliva and urine)

All patients were instructed on urine and saliva collections and received a sterile bottle for storage at the UNIP clinic premises.

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Volumes between 20 and 40 mL of urine were collected, and samples were stored at – 80º C until the time of analysis.[14]

Regarding saliva, patients were instructed not to eat pasty or hardened foods for 1 hour before collection, as well as not to consume alcoholic beverages for at least 12 hours prior to collection. They could only drink water and had to brush their teeth at least 2 hours before collection. Individuals in each group remained sitting upright, in an airy and calm environment, and the collection of unstimulated saliva was performed by sputum in a disposable plastic tube (Falcon®, Rio de Janeiro) according to the modified Navazesh method.[15] Patients were instructed to expectorate 3 mL of saliva in the plastic tubes and then they were hermetically sealed, transported and stored at -80°C until the time of analysis.[16]

Preparation and Metabolomic Analysis of Samples

The samples were prepared and analyzed in the Mass Spectrometry Laboratory of the Chemical Engineering Department of the Polytechnic School of the University of São Paulo - DEMPSTER Mass Lab. After derivatization and extraction they were injected into a gas chromatograph coupled to a mass spectrometer (GC-MS).

The analysis methodology was developed in the laboratory itself by the team of chemists, chemical engineers and dental surgeons involved in a previous study, by BARNES et al.[17]

Urine:

After thawing the samples at room temperature, 1000 µL of urine was transferred to a 1.5 mL plastic tube (Eppendorf, Germany), by means of an automatic micropipette (Biohit, 100 to 1000 µL, Brazil); The samples were dried in Speedvac apparatus (Centrivap concentrator, Labconco, Brazil) for a period of 2h, or until complete drying. After the drying process, the compounds were extracted (Figure 1), in two steps.

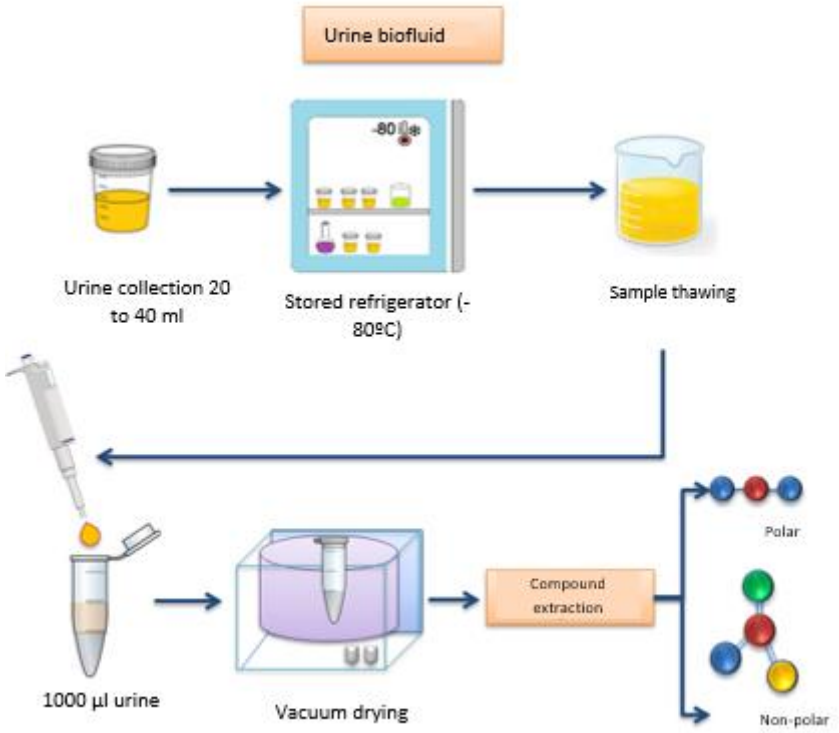

Figure 1. Collection, storage and drying of urine samples. Source: Alves, 2016.

In the first step, 1.0 mL of methanol was added to a plastic tube containing the dry sample, for the extraction of polar molecules, agitated in a vortex-type shaker (model AP59, Phoenic Luferco,

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3800 rpm, Brazil) for 2 minutes, and centrifuged in a laboratory centrifuge (model 800-1, 13000 rpm, Brazil) for 5 minutes; The supernatant product of this reaction was transferred to a new plastic tube, dried in a Speedvac apparatus and resuspended in 250 µL of methanol; The new supernatant product was transferred to a transparent 2 mL Vial tube (CMS 123TAT, Brazil) with stripe, for subsequent injection of 2 µL of the sample into a gas chromatograph coupled to a mass spectrometer (GC-MS, QP 2010 Plus, Shimadzu do Brasil), DB-1 column, 50 m long, internal diameter of 0.32 mm and film thickness of 1.20 µm (Figure 2).

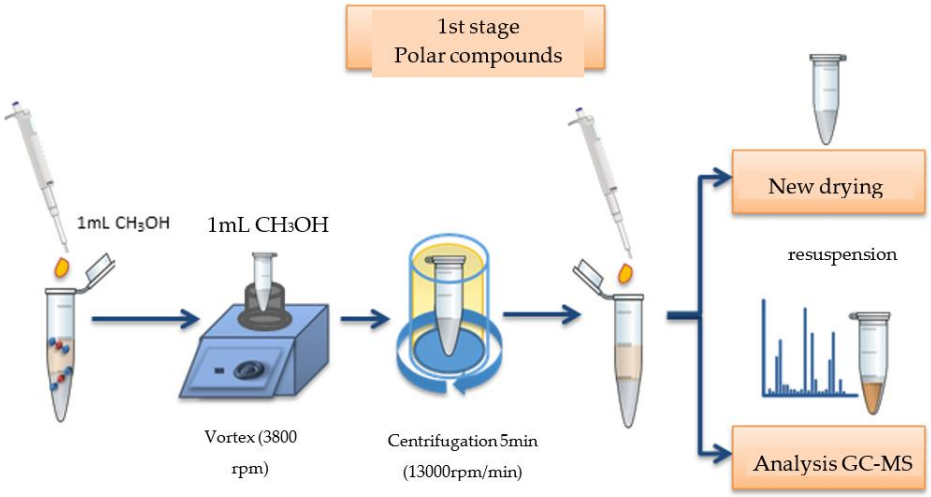

Figure 2. Process of extraction of metabolites from urine. Source: Alves, 2016.

The second stage consisted of a new extraction followed by derivatization with hexane to extract the nonpolar molecules present in the urine. 1 mL of hexane was added to the sample in the plastic tube and stirred on a vortex shaker for 2 seconds. 200 µL of 2 N methanolic sodium hydroxide solution was added and a new stirring was made. The tube was placed for 20 seconds in a bath at 50°C and stirred manually for one minute. 200 µL of methanolic hydrochloric acid (2N) solution was added and a new stirring in a vortex agitator was performed for 2 seconds, followed by centrifugation. There was the formation of two phases, the upper phase being the organic layer that contained esterified lipids solubilized in hexane. The supernatant product of this reaction was transferred to a new eppendorf, dried in Speedvac apparatus and resuspended in 250 µL of hexane. The new supernatant product was transferred to a Vial tube, via an electronic micropipette for further GC-MS analysis, under the same previous conditions (Figure 3).

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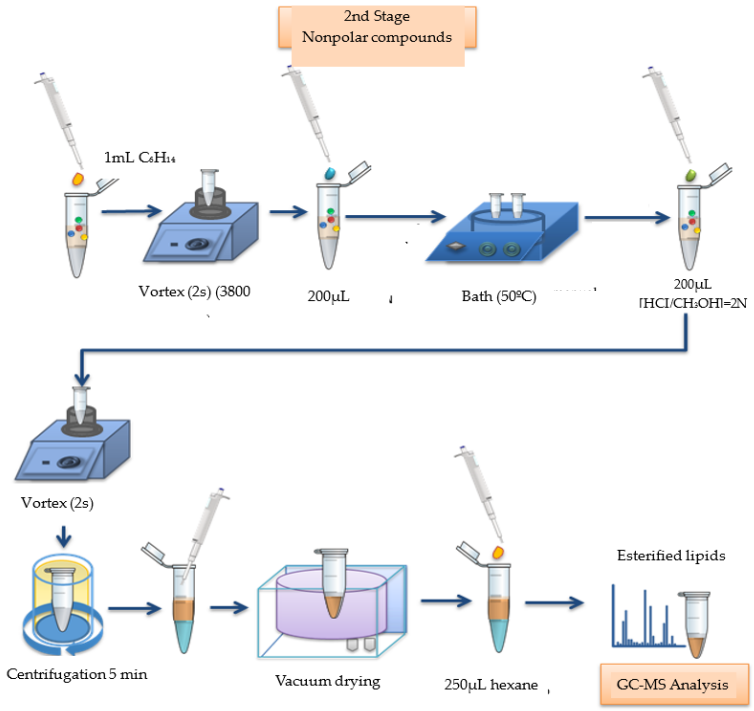

Figure 3. Derivatization process of urine metabolites. Source: Alves, 2016.

Spittle

First, 300 µL of saliva samples were dried in a vacuum centrifuge (Labconco Centrivap Concentrator, Kansas City, MI, USA). The metabolites were extracted by adding 300 µL of methanol containing methionine sulfonate as an internal standard and shaken (LCG Vortex Mixer, Taiwan, China) for 2 min, and the supernatant was dried in a vacuum centrifuge. After extraction, derivatization was performed by adding 100 µL of a solution with proportions (1:1) of N-methyl-N-(trimethylsilyl)trifluoroacetamide and a solvent solution: acetonitrile/dichloromethane/cyclohexane (5:4:1) and 5% trimethylamine. The samples were stirred for 30 s and then kept in a thermal bath (Nova Instruments NI 1225, Piracicaba, Brazil) at 60º C for 1 h. Then, the samples were centrifuged (Eppendorf Mini Spin, Hamburg, Germany) at 12,044 × g for 2 min. The supernatant was analyzed via GC-MS. The obtained data were processed using the GC MS solution and the metabolites identified using the smart metabolite database version 4.2 (Figure 4).

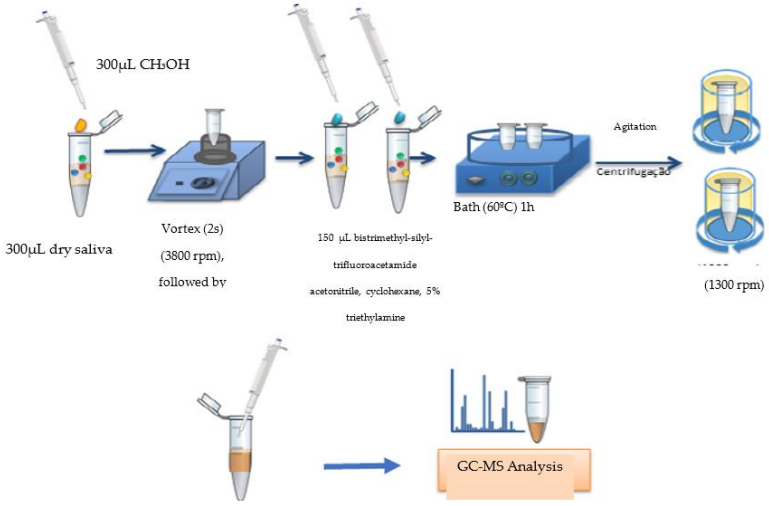

Figure 4. Process of extraction and derivatization of salivary metabolites. Source: Alves, 2016.

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GC-MS analysis conditions:

• Samples were analyzed under the following conditions:
Injector temperature: 280°C
Initial column temperature: 50°C
Heating rate: 5°C per min up to 300°C, remaining at this temperature for 10 min.
Total running time: 60 min
Ionization source temperature: 300°C
Solvent cut: 5 min
Interface temperature: 290°C
We obtained a total ion chromatogram (TIC) with mass range of 40 to 600 Da.

RESULTS

Sociodemographic Aspects and Clinical Parameters

Regarding the sociodemographic aspects, the patients in groups G1 and G2 totaled 32, being 90.5% male and 9.5% female. With regard to race, 62.5% were leukoderms and 37.5% were melanoderms. The age range of these groups ranged from 20 to 68 years (mean age 48 years). Regarding habits, 25% were ethyl, 6% were smokers and 13% were smokers/ethyl. Regarding comorbidities, it was found that 3.12% had Herpes Zoster, 3.12% Syphilis, 3.12% Hepatitis B and 3.12% Hepatitis C (Table 1).

In group G1 the probing depth ranged between 1mm (smallest pouch) and 3mm (largest pouch), without bleeding, and in group G2 the probing depth ranged between 4mm (smallest pouch) and 9mm (largest pouch), with bleeding (Table 1).

In groups G3 and G4, the total number of patients was 33, 48% male and 52% female. Of these patients, 79% were leukoderms and 21% were melanoderms. The age group of these groups ranged from 22 to 74 years (mean age 47 years); 12% reported being smokers and 18% reported having hypertension (Table 1).

In group G3 the probing depth ranged between 1mm (smallest pouch) and 3mm (largest pouch), without bleeding, and in group G4 the probing depth ranged between 3mm (smallest pouch) and 8mm (largest pouch), with bleeding (Table 1).

Table 1. Distribution and frequency of clinical variables in the study.

Variable	Absence of PD (N = 30)	Presence of PD (N = 35)
Age (Mean ± SD (N))	44.27 ± 14.02 (N = 30)	50.23 ±9.83 (N = 35)
Sex		
Female	11 (36.67%)	9(25.71%)
Male	19 (63.33%)	26 (74.29%)
Race		
Leukoderma	25 (83.33%)	21 (60%)
Melanoderma	5(16.67%)	14 (40%)
Habits		
Ethyl	5(16.67%)	3 (8.57%)
Smoker	1 (3.33%)	5(14.29%)
Ethyl/Smoker	1 (3.33%)	3 (8.57%)
None	23 (76.67%)	24 (68.57%)
Diseases		
HerpesZoster	1 (3.33%)	0 (0%)
Syphilis	1 (3.33%)	0 (0%)
Hepatitis B	0 (0%)	1 (2.86%)
Hepatitis C	0 (0%)	1 (2.86%)
Hypertension	1 (3.33%)	5(14.29%)

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None	27 (90%)	28 (80%)
Sounding depth		
< 3mm	27 (90%)	0 (0%)
3 to 4 mm	3 (10%)	8(22.86%)
> 4mm	0 (0%)	27 (77.14%)

Key: SD = Periodontal disease

Metabolomic analysis

Origin Pro and Statistica 12 software were used to generate the graphs and statistical analyses. We used the Anova techniques with Fisher's LSD test a *posteriori,* to compare the areas between the groups, variable that is directly proportional to the concentrations of each metabolite.

We built the Venn Diagram to identify the metabolites trivial to the groups and the unique metabolites. The chi-square test was also performed to compare single metabolites between groups at different time points.

Urine

The values in parentheses at the top of the graph are the degrees of freedom of the analysis and F is the F statistic.

Table 2. Means, standard deviation (SD), the lower and upper limits of the 95% confidence interval around the mean for metabolites present in urine.

Group	Grade	DP	Average - IC 95%	Average + IC 95%
G1	839049	163116.3	519268	1158831
G2	1584567	126826.4	1335930	1833204
G3	1040023	139487.8	766539	1313507
G4	1191700	141962.7	913364	1470037

In the urine analysis (Figure 5), we can observe that group 2 has the largest area. It is evident from the statistical analyses that there are significant differences between G1 and G2 (p =0.0003), with G2 being greater than G1. The area of G2 is greater than G3 (p =0.003) and greater than G4 (p =0.034). There are no significant differences between the areas of G3 and G4 (p =0.427), nor between G1 and G3 (p =0.341) and G1 and G4 (p =0.09). Evaluating the graph, the greater the degree of freedom (value above the graph), the greater the differences between the means, and the p value indicates that there is at least one difference between the means. The other p values are found with the a *posteriori* test, as it will indicate which means differ from each other, and it is only performed if the first p value is significant. For the case of urine, we found a value of p = 0.001 (p <0.05 considered the reference value for statistical significance), thus confirming the differences between groups.

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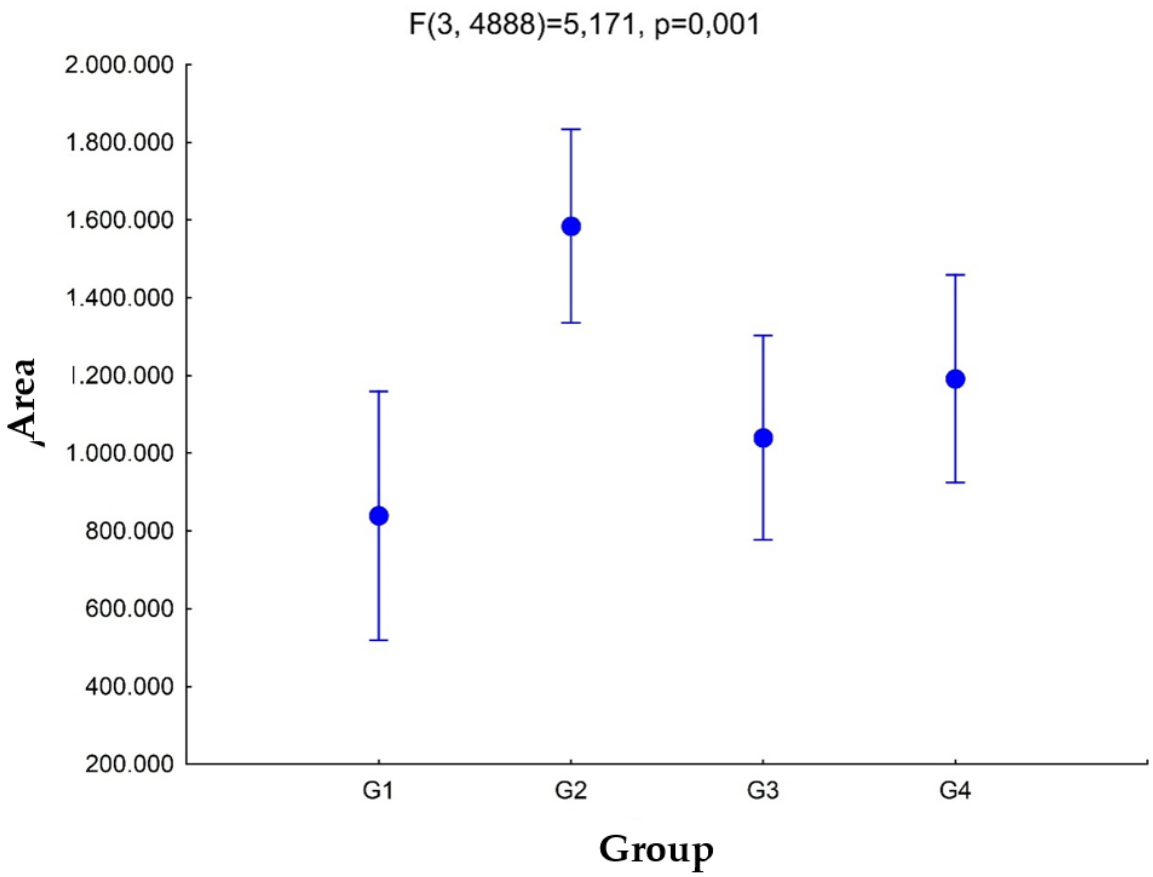

Figure 5. Anova comparing area averages of urine metabolites for the 4 groups. The bars denote a 95% confidence interval around the mean.

The 25 most representative metabolites were listed in terms of area in each group, and compared via a venn diagram (Figure 6). This analysis allowed identifying which metabolites were present in each group, and how many and which metabolites the groups shared with each other.

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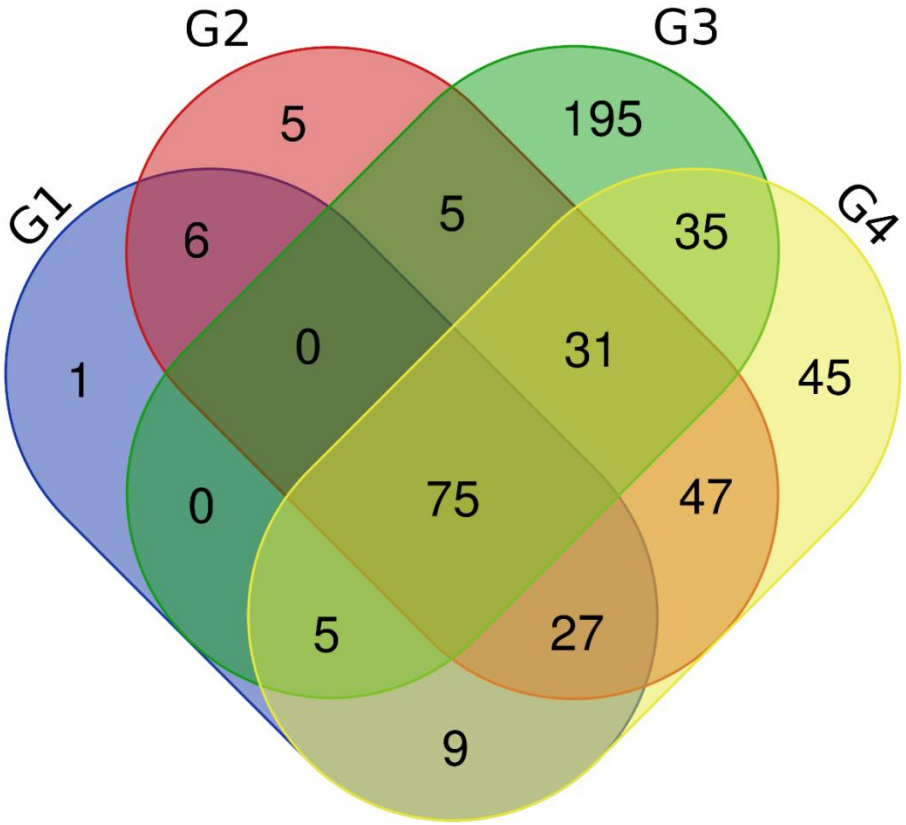

Figure 6. Venn diagram between groups for metabolites present in urine.

Table 3. Frequencies in the Venn diagram.

Group	Number of metabolites	Number of unique metabolites
G1	878	123
G2	1453	196
G3	1300	346
G4	1256	274
Number of unique metabolites		486

Spittle

The values in parentheses at the top of the graph are the degrees of freedom of the analysis and F is the F statistic.

Table 4. Means, standard deviation (SD), the lower and upper limits of the 95% confidence interval around the mean for metabolites present in urine.

Grupo	Média	DP	Média - IC 95%	Média + IC 95%
G1	1360668	270405,7	830472	1890864
G2	950794	229888,1	500042	1401545
G3	1052931	116612,6	824295	1281567
G4	1222791	117792,0	991843	1453740

Regarding saliva analysis (Figure 7) we can observe that group 1 has the largest area, however, from the statistical calculations there were no statistically significant differences between the areas of the groups (p =0.376). To test whether the differences in the tables in Appendix A were statistically

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significant, we used the Chi-square test and found that there are statistically significant differences between the number of unique metabolites in each group (X^2 =71.84, GL=3, p <0.00001).

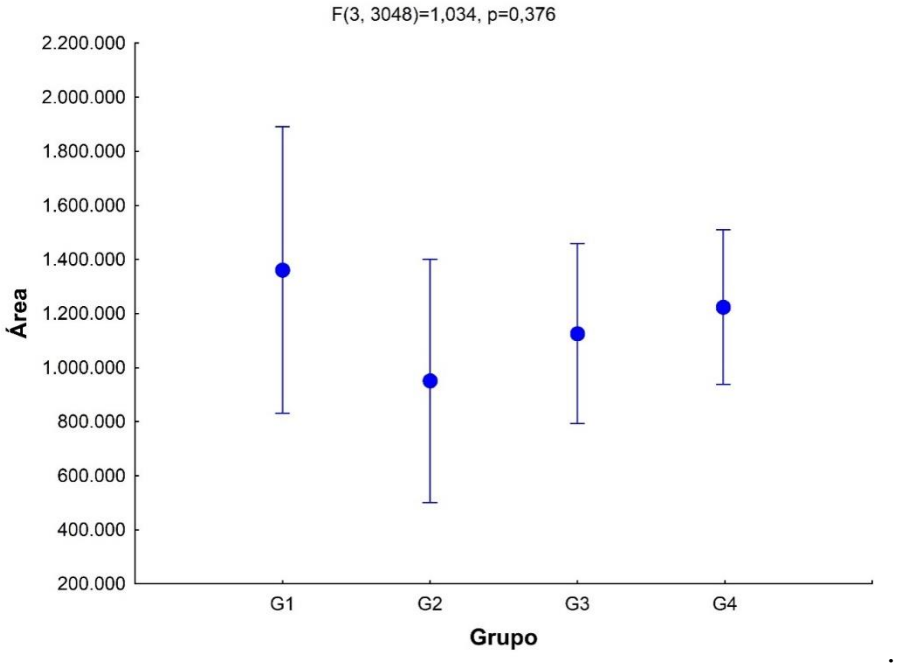

Figure 7. Anova comparing area averages of urine metabolites for the 4 groups. The bars denote a 95% confidence interval around the mean.

The 25 most representative metabolites were listed in terms of area in each group, and compared via a venn diagram (Figure 8). This analysis allowed identifying which metabolites were present in each group, and how many and which metabolites the groups shared with each other.

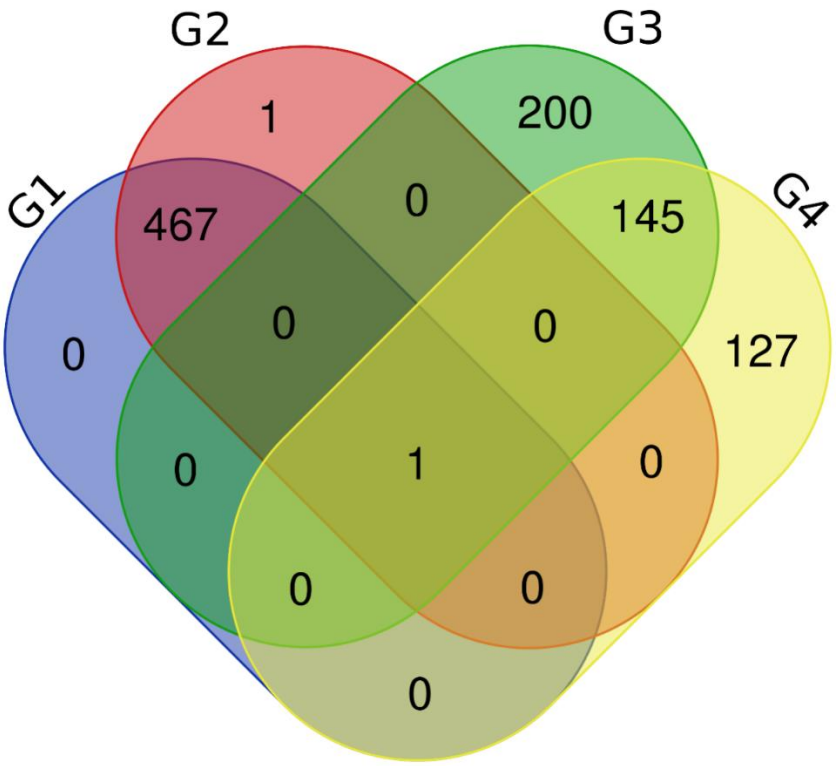

Figure 8. Venn diagram between groups for metabolites present in urine.

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Table 5. Frequencies in the Venn diagram.

Group	Number of metabolites	Number of unique metabolites
G1	1092	468
G2	2244	469
G3	1300	346
G4	1254	273
Number of unique metabolites		941

In urine, 4 metabolites are repeated in the 4 groups, identified in Table 6.

Table 6. Urine metabolites that are repeated in all groups of urine G1, G2, G3, G4.

URINA	
Metabólito	**Grupos**
Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	G1,G2,G3,G4
Ethanimidic acid, N-(trimethylsilyl)-, trimethylsilyl ester	G1,G2,G3,G4
Benzonitrile, 4-(2-methyl-1,3-dioxolan-2-yl)-	G1,G2,G3,G4
.alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	G1,G2,G3,G4

In saliva, 31 metabolites are repeated, but the metabolites that appear in groups with HIV (G1 and G2) do not appear in groups without HIV (G3 and G4), where G3 is the control group, therefore, 17 metabolites are repeated in groups G1 and G2 and 14 metabolites are repeated in groups G3 and G4. Identified in Table 7.

Table 7. Salivary metabolites from groups G1 and G2 that are repeated among themselves, and from groups G3 and G4 that are repeated among themselves.

SALIVA	
Metabólito	**Grupos**
Glucose-meto-5TMS(2)	G1,G2
1,6-Anhydroglucose-3TMS	G1,G2
Mannitol-6TMS	G1,G2
Citric acid-4TMS	G1,G2
Serine-3TMS	G1,G2
Isocitric acid-4TMS	G1,G2
Isoleucine-2TMS	G1,G2
Arabitol-5TMS	G1,G2
Vanilmandelic acid-3TMS	G1,G2
Threonic acid-4TMS	G1,G2
Cysteine-3TMS	G1,G2
Sucrose-8TMS	G1,G2
Palmitic acid-TMS	G1,G2
Threitol-4TMS	G1,G2
Ribonic acid-5TMS	G1,G2
Glucuronic acid-meto-5TMS(2)	G1,G2
2-Hydroxyglutaric acid-3TMS	G1,G2
.beta.-D-Galactofuranose, 1,2,3,5,6-pentakis-O-(trimethylsilyl)-	G3,G4
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-	G3,G4

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Talose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-	G3,G4
.beta.-D-Glucopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	G3,G4
D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-glucopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)-	G3,G4
D-Glucopyranose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-galactopyranosyl]-1,2,3,6-tetrakis-O-(trimethylsilyl)-	G3,G4
Maltose, octakis(trimethylsilyl)-	G3,G4
Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	G3,G4
Benzonitrile, 4-(2-methyl-1,3-dioxolan-2-yl)-	G3,G4
D-Turanose, heptakis(trimethylsilyl)-	G3,G4
.alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	G3,G4
2,4,6-Tri-t-butylbenzenethiol	G3,G4
Ethanimidic acid, N-(trimethylsilyl)-, trimethylsilyl ester	G3,G4
L-Proline, 1-(trimethylsilyl)-, trimethylsilyl ester	G3,G4

Discussion

Metabolomic analysis was applied in the present study to determine metabolites that corroborate possible associations between HIV/AIDS and periodontal disease (PD). of the diseases studied in the research and the opportunity to analyze the causal relationships between these metabolites and the evolution of both diseases, substantiating the bidirectional relationship between them, through the recognition of these metabolites, it was possible to estimate the variation in their concentrations, and through from systems biology analysis, to verify how these metabolic pathways were altered by both diseases.[8,18–20]

We analyzed biological samples (saliva and urine), which showed great variability, either due to changes in the metabolic processes of patients systemically compromised or even due to the use of medication, which favored the emergence of some atypical results observed in the analyses.

Barnes et al.[17] demonstrated that estimates of biofluid metabolites are important to draw a phenotypic profile of certain individuals in relation to the predisposition to manifest or not certain pathologies. Saude, Sykes [21], corroborate with the validation of studies the use of saliva and urine samples, due to the ease of collection and their nature rich in metabolites, which are constantly used as biofluids for research in humans and animals.

Studies such as that of Ryder et al. [22], show us the relevance of HIV/AIDS infection in relation to the increased risk of opportunistic infections, especially those associated with oral and periodontal disorders, with oral manifestations being the first clinical presentations and periodontal disease one of the main most prevalent oral disorders in patients infected with HIV/AIDS.

Patients who are on ART/HAART, due to the presence of antigens and activation of the immune system, are in a systemic inflammatory state, predisposing them to periodontal disease (PD). Microbiota-derived metabolites influence molecular and cellular processes and modulate immune system maturation at the tissue level. Inflammatory and immunological reactions correspond to most of the tissue lesions observed in PD.[23–25] The results observed (Figures 5 and 7), corroborate these statements, as they demonstrate that groups 1 and 2 have larger areas of metabolites in relation to their concentrations, and most are fatty acids, which are precursors of arachidonic acid, an important anti-inflammatory agent. In salivary analysis, among the 25 most significant metabolites, palmitic acid was found to be present, which shows that perhaps, patients living with HIV/AIDS (PVHIV/AIDS) because they are under art/HAART, may have a higher expression of this metabolite in the inflammatory pathways of the body, it was observed in higher concentration in PVHIV/AIDS and with PD, (G2).

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Corroborating the studies of Çiçek et al.[26], our results showed that the concentration of poly and monounsaturated carboxylic acids presents as altered in inflamed gingival tissues (G1 and G2).

Another metabolite in high concentration was proline, which is associated with collagen degradation and is shown to inhibit metalloproteinases. Some microorganisms present in periodontal lesions are more proteolytic than others and can produce these amino acids. According to Barnes et al.[17] and Luchian et al.[27], is the imbalance between the active form of matrix metalloproteins and their endogenous inhibitors that promote pathological collapse of the extracellular matrix. In addition, the fact that some patients presented comorbities and are immunocompromised shows that some metabolites found, such as proline, may also be related to the systemic diseases and not only to periodontal disease [13]. Some individuals were smokers, ethyl/smokers and presented high blood pressure, and the effect of this occurrence is much more related to the presence of oral lesions rather than the type and amount of metabolites found.

One fact that was quite highlighted in groups 3 and 4, is that all 25 of the most significant metabolites present in saliva were also present in urine. From this data it can be inferred that because they are groups of patients not diagnosed with HIV/AIDS, no metabolic process suppresses or enhances the formation of more metabolites. However, as for the quantification of each of them, it is possible to verify differences in their concentrations.

Finally, some groups did not show results with large statistical differences, given that the sample number was small with a calculation of sample power of 68%, this was due to the fact that all collections were carried out during the COVID 19 pandemic period, and because we have a sample classified as a risk population, also the number of metabolites found was large spectrum and we only selected the 25 with the highest concentration for each group. It is suggested, therefore, that the continuity of this study happens, thus increasing the number of participants in each group, also the possibilities of new discoveries.

Conclusions

We concluded that from the metabolomic analysis it was possible to distinguish the biomarkers of patients living with HIV/AIDS with periodontal disease and without periodontal disease;

Some metabolites such as amino acids, carboxylic acids and fatty acids were over expressed in patients living with HIV/AIDS and periodontal disease;

Most metabolites in the carbohydrate group were under expressed in patients living with HIV/AIDS, showing that even in systemic compensation situations these patients have a different metabolic profile.

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Conflicts of Interest: Authors declare no conflict of interest.

Appendix A - Urine

25 most representative metabolites in terms of area in each urine group

GROUP G1

Table 7. The 25 metabolites with the largest area in the G1 group, p<0.0001 (i.e., there are statistically significant differences).

Metabólito	Média	DP
1,3,5-Triazine, 2,4,6-tris[(trimethylsilyl)oxy]-	8865952	710467

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Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	6207136	653534
N-Ethyl-5-propyl-5-nonanamine	5473310	1666193
Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	4234076	745144
Glycine, N-(trimethylsilyl)-, trimethylsilyl ester	4222151	710467
Ethanimidic acid, N-(trimethylsilyl)-, trimethylsilyl ester	3913242	1360441
Bis(trimethylsilyl)monostearin	3646899	745144
L-Proline, 1-(trimethylsilyl)-, trimethylsilyl ester	3594686	745144
2,4,6-Tri-t-butylbenzenethiol	2639443	1666193
Benzonitrile, 4-(2-methyl-1,3-dioxolan-2-yl)-	2623687	462119
D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-galactopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)-	2623670	1666193
Urea, N,N'-bis(trimethylsilyl)-	2614503	453480
l-Alanine, N-(trimethylsilyl)-, trimethylsilyl ester	2521168	785451
Octadecanoic acid, trimethylsilyl ester	2429429	745144
Glucose, pentakis-O-trimethylsilyl-	2187543	1666193
Pentanoic acid, 5-[bis(trimethylsilyl)amino]-, trimethylsilyl ester	1907578	785451
1-(3-Methylbutyl)-2,3,4,6-tetramethylbenzene	1468944	1053793
3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionic acid	1407772	1053793
.alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	1350668	555398
L-Valine, N-(trimethylsilyl)-, trimethylsilyl ester	1059684	833096
3-Diazo-1-methyl-1,3-dihydro-indol-2-one	978722	235652

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.beta.-L-Mannopyranose, 6-deoxy-1,2,3,4-tetrakis-O-(trimethylsilyl)-	792553	480988
Glucopyranose pentaTMS	791134	1666193
Hexadecanoic acid, trimethylsilyl ester	790838	745144
Benzene, 1-(1,3-dimethyl-3-butenyl)-4-fluoro-	744349	1053793

GROUP 2

Table 8. The 25 metabolites with the largest area in the G2 group, p<0.0001.

Metabólito	Média	DP
D-Glucopyranose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-galactopyranosyl]-1,2,3,6-tetrakis-O-(trimethylsilyl)-	54130868	3646427
Talose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-	43096150	3646427
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-	32580927	2578413
.beta.-D-Galactofuranose, 1,2,3,5,6-pentakis-O-(trimethylsilyl)-	27413320	3646427
.beta.-D-Glucopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	26772088	2578413
D-Mannopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	21864900	3646427
Arabinofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-	20942458	3646427
D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-galactopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)-	15713470	2105265
Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	14436441	1153101
Maltose, octakis(trimethylsilyl)-	12848208	3646427
D-Fructose, 6-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.alpha.-D-glucopyranosyl]-1,3,4,5-tetrakis-O-(trimethylsilyl)-	11735572	3646427
D-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-	10441678	1718942
D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-glucopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)-	9125283	3646427
1,3,5-Triazine, 2,4,6-tris[(trimethylsilyl)oxy]-	8417359	1183057

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	Acrylic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	5871478	3646427
	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	4725717	1250714
	Bis(trimethylsilyl)monostearin	4703488	1331487
	Benzonitrile, 4-(2-methyl-1,3-dioxolan-2-yl)-	4516857	897688
	Ethanimidic acid, N-(trimethylsilyl)-, trimethylsilyl ester	4203803	3646427
	.alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	3834960	795716
	D-Turanose, heptakis(trimethylsilyl)-	3732689	1250714
	Urea, N,N'-bis(trimethylsilyl)-	2828885	701755
	Octadecanoic acid, trimethylsilyl ester	2211092	1183057
	Glucose, pentakis-O-trimethylsilyl-	2187543	2578413
	Glycine, N-(trimethylsilyl)-, trimethylsilyl ester	2140387	1488647

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GROUP 3

Table 9. The 25 metabolites with the largest area in the G3 group, p<0.0001.

Metabólito	Média	DP
.beta.-D-Galactofuranose, 1,2,3,5,6-pentakis-O-(trimethylsilyl)-	64909445	3638542
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-	50559713	2572838
Talose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-	45271460	3638542
.beta.-D-Glucopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	43726768	2100713
D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-glucopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)-	38610758	2572838
Hexopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	24080346	3638542
D-Glucopyranose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-galactopyranosyl]-1,2,3,6-tetrakis-O-(trimethylsilyl)-	15958348	2100713
Inososе-2, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, myo-	15513174	3638542
D-Ribofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-	13662867	3638542
Maltose, octakis(trimethylsilyl)-	13546098	2572838
Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	10854091	1097062
Dihydroxyacetone dimer, tetra(trimethylsilyl)-	7785312	3638542
D-Xylopyranose, 1,2,3,4-tetrakis-O-(trimethylsilyl)-	7544071	3638542
(.+/-.)-2,3-Butanediol diTMS	6635889	3638542
Benzonitrile, 4-(2-methyl-1,3-dioxolan-2-yl)-	6302249	758688
.alpha.-L-Galactofuranose, 6-deoxy-1,2,3,5-tetrakis-O-(trimethylsilyl)-	5392053	3638542
D-Turanose, heptakis(trimethylsilyl)-	5341013	1009150
Phenol, 2,4-bis(1,1-dimethylethyl)-	4607213	2572838

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Glucofuranoside, methyl 2,3,5,6-tetrakis-O-(trimethylsilyl)-, .alpha.-D-	4213623	2572838
.alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	4080467	813603
2,4,6-Tri-t-butylbenzenethiol	2836874	1375240
Ethanimidic acid, N-(trimethylsilyl)-, trimethylsilyl ester	2836511	1819271
Ethylbis(trimethylsilyl)amine	2601776	3638542
L-Proline, 1-(trimethylsilyl)-, trimethylsilyl ester	2498879	1819271
2,6-Octadiene, 3,7-dimethyl-1-(hydroxydimethylsilyl)-1-(trimethylsilyl)-	2206954	3638542

GROUP 4

Table 10. The 25 metabolites with the largest area in the G4 group, p<0.0001.

Metabólito	Média	DP
D-Glucopyranose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-galactopyranosyl]-1,2,3,6-tetrakis-O-(trimethylsilyl)-	54130868	4002282
Talose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-	43096150	4002282
D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-galactopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)-	41893070	4002282
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-	32580927	2830041
.beta.-D-Galactofuranose, 1,2,3,5,6-pentakis-O-(trimethylsilyl)-	27413320	4002282
.beta.-D-Glucopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	26772088	2830041
D-Mannopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	21864900	4002282
Arabinofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-	20942458	4002282
Maltose, octakis(trimethylsilyl)-	12848208	4002282
D-Fructose, 6-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.alpha.-D-glucopyranosyl]-1,3,4,5-tetrakis-O-(trimethylsilyl)-	11735572	4002282
D-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-	9490839	1789875

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Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	9218777	1069655
D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-glucopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)-	9125283	4002282
1,3,5-Triazine, 2,4,6-tris[(trimethylsilyl)oxy]-	8596650	1033385
D-Turanose, heptakis(trimethylsilyl)-	7561628	2001141
.alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	6501577	1155359
Acrylic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	5871478	4002282
Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	5513926	1033385
Benzonitrile, 4-(2-methyl-1,3-dioxolan-2-yl)-	4065195	743205
Bis(trimethylsilyl)monostearin	3612728	1069655
2,4,6-Tri-t-butylbenzenethiol	3550449	2310719
Glucose, pentakis-O-trimethylsilyl-	2902541	4002282
Ethanimidic acid, N-(trimethylsilyl)-, trimethylsilyl ester	2629166	2830041
1H-Indole-3-methanamine, N,N-dimethyl-	2593132	4002282
L-Proline, 1-(trimethylsilyl)-, trimethylsilyl ester	2582950	1415021

Appendix B - Spittle

25 most representative metabolites in terms of area in each saliva group

GROUP 1

Table 11. The 25 metabolites with the largest area in the G1 group, p=0.993 (i.e., there are no statistically significant differences).

Metabólito	Média	DP
Galactose-meto-5TMS(2)	28932614	4670991
Glucose-meto-5TMS(2)	28614032	4670991
Isomaltose-meto-8TMS(2)	17457895	4670991
1,6-Anhydroglucose-3TMS	15798295	4670991
Glycine-3TMS	13662378	4670991

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Mannitol-6TMS	13361370	4670991
Citric acid-4TMS	11716427	4670991
Serine-3TMS	11041226	3813848
Isocitric acid-4TMS	10433067	6605779
Isoleucine-2TMS	9334604	4670991
Arabitol-5TMS	7824703	4670991
Vanilmandelic acid-3TMS	5070400	6605779
Threonic acid-4TMS	5030182	4670991
Glycine-2TMS	4960489	3813848
2-Aminoethanol-3TMS	4061667	3813848
Cysteine-3TMS	3912423	4670991
Sucrose-8TMS	3640156	4670991
Palmitic acid-TMS	3057804	4670991
4-Hydroxyphenyllactic acid-3TMS	3035529	6605779
Threitol-4TMS	2941068	4670991
2-Deoxy-glucose-4TMS(1)	2783354	4670991
Ribonic acid-5TMS	2650150	6605779
Alanine-2TMS	2616988	6605779
Glucuronic acid-meto-5TMS(2)	2598801	6605779
2-Hydroxyglutaric acid-3TMS	2564823	4670991

GROUP 2

Table 12. The 25 metabolites with the largest area in the G2 group, p=0.999, there are no statistically significant differences.

Metabólito	Média	DP
Proline-2TMS	28353197	5674798
Mannitol-6TMS	16556688	2537847
1,6-Anhydroglucose-3TMS	13358231	2537847
Glucose-meto-5TMS(2)	13022293	2837399
Palmitic acid-TMS	8594207	3276346
Citric acid-4TMS	6910706	2837399
Leucine-2TMS	6263040	3276346
Cadaverine-3TMS	5831356	2837399
Isocitric acid-4TMS	5369195	4012688
Vanilmandelic acid-3TMS	5084182	5674798
2-Hydroxyglutaric acid-3TMS	3877160	5674798
Serine-3TMS	3833007	3276346
Indol-3-acetic acid-2TMS	3719565	5674798
Threonic acid-4TMS	3291717	3276346
Aconitic acid-3TMS	3193806	5674798
meso-Erythritol-4TMS	2222647	3276346

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	Arabitol-5TMS	2075037	2537847
	Sucrose-8TMS	2074208	2837399
	Isoleucine-2TMS	2014361	3276346
	Cysteine-3TMS	1973099	2837399
	Threitol-4TMS	1911564	3276346
	Threonine-3TMS	1525751	4012688
	4-Hydroxyphenylacetic acid-2TMS	1353572	5674798
	Glucuronic acid-meto-5TMS(2)	1297058	4012688
	Ribonic acid-5TMS	1296552	4012688

GROUP 3

Table 13. The 25 metabolites with the largest area in the G3 group, p<0.0001.

Metabólito	Média	DP
.beta.-D-Galactofuranose, 1,2,3,5,6-pentakis-O-(trimethylsilyl)-	64909445	3638542
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-	50559713	2572838
Talose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-	45271460	3638542
.beta.-D-Glucopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	43726768	2100713
D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-glucopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)-	38610758	2572838
Hexopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	24080346	3638542
D-Glucopyranose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-galactopyranosyl]-1,2,3,6-tetrakis-O-(trimethylsilyl)-	15958348	2100713
Inososе-2, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, myo-	15513174	3638542
D-Ribofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-	13662867	3638542
Maltose, octakis(trimethylsilyl)-	13546098	2572838
Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	10854091	1097062
Dihydroxyacetone dimer, tetra(trimethylsilyl)-	7785312	3638542
D-Xylopyranose, 1,2,3,4-tetrakis-O-(trimethylsilyl)-	7544071	3638542

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(.+/-.)-2,3-Butanediol diTMS	6635889	3638542
Benzonitrile, 4-(2-methyl-1,3-dioxolan-2-yl)-	6302249	7586808
.alpha.-L-Galactofuranose, 6-deoxy-1,2,3,5-tetrakis-O-(trimethylsilyl)-	5392053	3638542
D-Turanose, heptakis(trimethylsilyl)-	5341013	1009150
Phenol, 2,4-bis(1,1-dimethylethyl)-	4607213	2572838
Glucofuranoside, methyl 2,3,5,6-tetrakis-O-(trimethylsilyl)-, .alpha.-D-	4213623	2572838
.alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	4080467	813603
2,4,6-Tri-t-butylbenzenethiol	2836874	1375240
Ethanimidic acid, N-(trimethylsilyl)-, trimethylsilyl ester	2836511	1819271
Ethylbis(trimethylsilyl)amine	2601776	3638542
L-Proline, 1-(trimethylsilyl)-, trimethylsilyl ester	2498879	1819271
2,6-Octadiene, 3,7-dimethyl-1-(hydroxydimethylsilyl)-1-(trimethylsilyl)-	2206954	3638542

GROUP 4

Table 14. The 25 metabolites with the largest area in the G4 group, p<0.0001.

Metabólito	Média	DP
D-Glucopyranose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-galactopyranosyl]-1,2,3,6-tetrakis-O-(trimethylsilyl)-	54130868	4002282
Talose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-	43096150	4002282
D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-galactopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)-	41893070	4002282
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-	32580927	2830041
.beta.-D-Galactofuranose, 1,2,3,5,6-pentakis-O-(trimethylsilyl)-	27413320	4002282
.beta.-D-Glucopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	26772088	2830041

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D-Mannopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	21864900	4002282
Arabinofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-	20942458	4002282
Maltose, octakis(trimethylsilyl)-	12848208	4002282
D-Fructose, 6-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.alpha.-D-glucopyranosyl]-1,3,4,5-tetrakis-O-(trimethylsilyl)-	11735572	4002282
D-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-	9490839	1789875
Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	9218777	1069655
D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-glucopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)-	9125283	4002282
1,3,5-Triazine, 2,4,6-tris[(trimethylsilyl)oxy]-	8596650	1033385
D-Turanose, heptakis(trimethylsilyl)-	7561628	2001141
.alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	6501577	1155359
Acrylic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	5871478	4002282
Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	5513926	1033385
Benzonitrile, 4-(2-methyl-1,3-dioxolan-2-yl)-	4065195	743205
Bis(trimethylsilyl)monostearin	3612728	1069655
2,4,6-Tri-t-butylbenzenethiol	3550449	2310719
Glucose, pentakis-O-trimethylsilyl-	2902541	4002282
Ethanimidic acid, N-(trimethylsilyl)-, trimethylsilyl ester	2629166	2830041
1H-Indole-3-methanamine, N,N-dimethyl-	2593132	4002282
L-Proline, 1-(trimethylsilyl)-, trimethylsilyl ester	2582950	1415021

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