DANIELE ASSAD SUZUKI

SALIVARY BIOMARKERS FOR CANCER DIAGNOSIS

BIOMARCADORES SALIVARES PARA O DIAGNÓSTICO DE CÂNCER

BRASÍLIA, 2019

UNIVERSIDADE DE BRASÍLIA

FACULDADE DE CIÊNCIAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

DANIELE ASSAD SUZUKI

SALIVARY BIOMARKERS FOR CANCER DIAGNOSIS

BIOMARCADORES SALIVARES PARA O DIAGNÓSTICO DE CÂNCER

Thesis presented as partial requirement for achievement the degree of Doctor in Medical Fisiopathology in the Program of Postgraduate Health Sciences of University of Brasilia.

Tese apresentada como requisito parcial para obtenção do título de Doutor em Fisiopatologia Médica pelo programa de Pós-Graduação em Ciências da Saúde da Universidade de Brasília.

Advisor: Prof. Dra. Eliete Neves da Silva Guerra

Co-Advisor: Prof. Dra. Ana Carolina Acevedo Poppe

Brasília

2019

DANIELE ASSAD SUZUKI

SALIVARY BIOMARKERS FOR CANCER DIAGNOSIS

BIOMARCADORES SALIVARES PARA O DIAGNÓSTICO DE CÂNCER

Thesis presented as partial requirement for achievement the degree of Doctor in Medical Fisiopathology in the Program of Postgraduate Health Sciences of University of Brasilia.

Aprovado em 14 de outubro de 2019

BANCA EXAMINADORA

Prof. Dra. Eliete Neves da Silva Guerra (presidente)

Universidade de Brasília (UNB)

Prof. Dra Adriana Lofrano Alves Porto

Universidade de Brasília (UNB)

Prof. Dra. Audrey Coombes

École supérieure de physique et de chimie industrielles de la Ville de Paris

Prof. Dr. Max Senna Mano

IEP- Instituto de Ensino e Pesquisa do Hospital Sírio Libanês

To my beloved and unforgettable father, Luiz Antonio Assad, who taught me all the values to pursuit a path in the right way. To my mother Elina Assad, who once told me that if I studied really hard, I could be anything I wanted.

ACKNOWLEDGMENTS

To Universidade de Brasília;

To Fundação de Apoio à Pesquisa do Distrito Federal –FAPDF;

To Laboratório de Histopatologia Bucal- UnB;

To Laboratório Interdisciplinar de Biociências da Faculdade de Medicina da UnB;

To Laboratório de Farmacologia Molecular –UnB;

To the Department of Analytical, Bioanalytical Sciences and Miniaturization -ESPCI- Paris;

To DIM analytics- Ile de France, France;

To Hospital de Base do Distrito Federal;

To Hospital Universitário de Brasília;

To Laboratório Sabin Medicina Diagnóstica;

To Hospital Sírio Libanês- DF;

To Cettro-Centro de Câncer de Brasília;

To Hélène Chardin;

To Audrey Combes;

To Valérie Pichon;

To Gustavo Barra;

To Ricardo Pratesi;

To Yanna Nobrega;

To Ludmila Thommen;

To Tatiana Strava.

Firstly, I would like to thank God and Our Lady for all the graces bestowed and the strength to complete this work.

To all my family, especially my husband Mauro for such understanding and love in these overburdened years. To my beloved daughter Maria Esther, whose existence already drives me to be a better person. To my brothers for all the positive vibes in my career.

To my dear laboratory colleagues, for the whole partnership and especially to Elisa Porto and Ana Gabriela Normando, who were essential in the doctorate and spared no effort to help me in any situation. To Gabriel Borges, an example of kindness and brilliance. To Gustavo Fernandes, my boss at Hospital Sírio Libanês, for all the encouragement in these years. To the colleagues at the Hospital Sírio Libanês for their collaboration, in particular to Igor Morbeck and Tatiana Strava. To Ludmila Thommen for her partnership and collaboration at Hospital Universitário de Brasília.

All my friends, for the fellowship and support at the times I needed you most, you know who you are.

To the members of the doctoral evaluation board, Dr Max Mano, Dr. Audrey Coombes, Dr. Caroline Lourenço and Dr. Adriana , for honoring me with your participation and contributions.

To my co-advisor Ana Carolina Poppe, for mentoring and advice. Besides being a great researcher, she is an excellent person with excelent humour.

To my mentor teacher Eliete Guerra, for investing in a doctor she didn't know and believing in me. An example of persistence and dedication, I will take your teachings forever.

To the University of Brasilia, my home, of which I am so proud. It is an honor to have completed my graduation, masters and now PhD here.

If you can dream it, you can do it.

(Walt Disney)

ABSTRACT

Excluding non-melanoma skin cancer, breast cancer (BC) is the most common type of cancer. The objectives of this study were: 1) to evaluate salivary concentration of cancer antigen 15-3 (CA15-3) in BC patients and healthy controls (HC) by chemiluminescence assay (CLIA), electrochemiluminescence assay (ECLIA) and enzyme-linked immunosorbent assay (ELISA) and to correlate with serum CA15 -3 determined by ECLIA; 2) to evaluate the diagnostic capacity of saliva metabolites in cancer patients in a systematic literature review; 3) determine the profile of metabolites in saliva of BC patients and HC by liquid chromatography followed by mass spectrometry. ELISA and CLIA, unlike ECLIA, were able to detect CA15-3 in the participants' saliva. The results were presented in the format of articles There was no significant difference between serum and salivary CA15-3 values in cases or HC. There was a moderate correlation between serum salivary CA15-3 levels in BC patients by ELISA ($r = 0.56$, $p = 0.0047$). The systematic review identified 1,151 studies and 25 were included. Most evaluated patients with breast and oral cavity cancer. 140 salivary metabolites were significant different between patients and HC, most of them amino acids. Mass spectrometry identified 534 ions in both groups. 31 ions were overexpressed in BC cases ($p < 0.05$). The Metlin database identified 7 oligopeptides and 6 glycerophospholipids (PG14:2, PA32:1, PS28:0, PS40:6, PI31:1 and PI38:7). Saliva is a promising diagnostic medium and BC metabolome studies can identify potential salivary biomarkers.

Keywords: breast cancer, CA15-3, saliva, metabolites, mass spectrometry.

RESUMO

Excluindo câncer de pele não melanoma, o câncer de mama é o tipo mais comum de neoplasia. Os objetivos desse estudo foram: 1) avaliar a concentração na saliva do antigeno do câncer 15-3 (CA15-3), em pacientes com câncer de mama e controles saudáveis, pelos métodos de quimioluminescência (CLIA), ensaio imunoenzimático (ELISA) e eletroquimioluminescência (ECLIA) e correlacionar com o CA15-3 sérico determinado por ECLIA; 2) avaliar a capacidade diagnóstica de metabólitos na saliva em pacientes com câncer em uma revisão sistemática da literatura; 3) determinar o perfil de metabólitos na saliva de pacientes com câncer de mama e controles saudáveis, através de cromatografia líquida seguida por espectometria de massa. Os resultados foram apresentados na forma de artigos. ELISA e CLIA, ao contrário de ECLIA, foram capazes de detectar CA15-3 na saliva das participantes. Não houve diferença significativa entre os valores médios séricos e salivares de CA15-3 em casos ou controles saudáveis. Houve uma correlação moderada entre os níveis séricos de CA15-3 salivar medido pelo ELISA em pacientes com câncer de mama ($r = 0,56$, $p = 0,0047$). A revisão sistemática identificou 1.151 estudos e 25 foram incluídos. A maioria avaliou pacientes com câncer de mama e de cavidade oral. 140 metabólitos salivares foram significativos entre pacientes e controles, sendo a maioria aminoácidos. Espectometria de massa identificou 534 íons nos dois grupos. 31 íons estavam superexpressos nos casos de câncer de mama ($p < 0,05$). A base de dados Metlin identificou 7 oligopeptídeos e 6 glicerofosfolípideos (PG14:2, PA32:1, PS28:0, PS40:6, PI31:1 e PI38:7). Saliva é um meio diagnóstico promissor e estudos de metaboloma em câncer de mama podem identificar potenciais biomarcadores salivares.

Palavras chave: câncer de mama, CA15-3, saliva, metabólitos, espectometria de massa.

LIST OF FIGURES

LIST OF TABLES

Article 3

LIST OF ABBREVIATIONS AND ACRONYMS

INCA: Brazilian National Cancer Institute

PSA: prostatic specific antigen

CA 125: cancer antigen 125

CA15-3: cancer antigen 15-3

CA19-9: cancer antigen 19-9

MUC1: mucin 1

ELISA: Enzyme-Linked Immunosorbent Assay

ATP : adenosine triphosphate

PI3K: phosphoinositide-3-kinase

TCA: tricarboxylic acid

CoA: coenzyme A

BCAA: branched-chain amino acid

ETC: electron transport chain

AA: amino acids

MW: molecular weight

MS: mass spectrometry

NMR: nuclear magnetic resonance spectroscopy

GC: gas chromatography

LC: liquid chromatography

CE: capillary electrophoresis

OXPHOS: oxidative phosphorylation

ROS: reactive oxygen species.

GSH: glutathione

LC/MS: liquid chromatography coupled with mass spectrometry

OC: oral cancer

BC: breast cancer

AUC: area under curve

LysoPC: lysophosphatidylcholine

MG: monoacylglycerol

CLIA: chemiluminescence assay

ECLIA: electrochemiluminescence assay

MUC: mucin

HUB: Hospital Universitário de Brasília

HBDF: Hospital de Base do Distrito Federal

Cettro: Centro de Câncer de Brasília

Rpm: rotations per minute

TPA: tripropylamine

RLU: relative light units

TN: triple negative

DSCF: Dwass, Steel, Critchlow-Fligner

ER: estrogen receptor

DTA diagnostic test accuracy

DNA: deoxyribonucleic acids

RNA: ribonucleic acids

SA: sialic acid or N-acetylneuraminate

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PROSPERO: prospective register of systematic reviews

HC: healthy control

HMDB: Human Metabolome Database

QUADAS- Quality Assessment of Diagnostic Accuracy Studies

CE-MS: capillary electrophoresis mass spectrometry

EIA: enzyme-immunoassay

HPLC: high performance liquid chromatography

RIA: radioimmunoassay

HPLC/MS : High Performance Liquid Chromatography-Mass Spectrometry

PC: pancreatic cancer

N: number

K: cases of cancer

SFAA: salivary free amino acid

CE/TOFMS: Capillary Electrophoresis Time-of-Flight Mass Spectrometry

SERS: surface- enhanced Raman spectroscopy

UPLC-QTOFMS :Ultra-Performance Liquid Chromatography–Mass Spectrometry coupled with Quadrupole/Time-of-Flight Mass Spectrometry

3PG: glyceraldehyde 3-phosphate

Trp: Tryptophan

Ru5P: ribulose 5-phosphate

F6P: fructose 6-phosphate

5RP:ribose 5-phosphate

SAM: S-adenosylmethionine

β-Ala: β-Alanine

Glu: glutamic acid

DHAP: dihydroxyacetone phosphate

Asp: aspartic acid

LTB4: Leukotriene B4

PGE2: prostaglandin E2

HETE: hydroxyeicosatetranoic acids

HNC: head and neck cancer

SPM: spermine

CAD: cadaverine

NACT: neo-adjuvant chemotherapy

AC: doxorrubicin plus cyclophosphamide

CR: complete response

PR: partial response

Ac-SPM: N1-acetyl-spermine

N1-Ac-SPD: N1-acetyl-spermidine

N8-Ac-SPD: N8-acetyl-spermidine

TSA: total silica acid

TP: total protein

LR: likelihood ratio

DOR: diagnostic odds ratio

N/D: not described in HMDB

N/F: not found in any database

PPV = positive predictive value

NPV = negative predictive value

LR+ = positive likelihood ratio

LR- = negative likelihood ratio

Pro = Proline

Thr = Threonine

His = Histidine.

HER2: human epidermal growth factor receptor 2

PR: progesterone receptor

UHPLC-Q-TOF/MS: Ultra high performance liquid chromatography quadrupole time-of-flight mass spectrometry

ROC: Receiver operating characteristic

PA: phosphatidic acid

PS: phosphatidylserine

PI : phosphatidylinositol

PG: phosphatidylglycerol

SUMMARY

1 INTRODUCTION

Worldwide, in 2018, there was an estimative of 2.1 million newly diagnosed breast cancer cases, accounting for almost 1 in 4 cancer cases among woman and 627,000 deaths (1). Excluding non-melanoma skin cancers, breast cancer is the most common type of cancer and the leading cause of cancer death among women(1). According to 2018 estimates from the Brazilian National Cancer Institute (INCA), 59,700 new cases would be diagnosed this year, with an estimated risk of 56.33 cases for each 100,000 women(2). In 2017, there were 16,927 deaths from breast cancer in Brazil(3).

Breast cancer is an extremely heterogeneous disease caused by interactions between inherited and environmental risk factors that lead to the progressive accumulation of genetic and epigenetic changes in breast cells(4). The most important risk factors are age over 40, history of mammary gland diseases, history of cancer in first-degree relatives, early menarche and late childbearing (after 35 years of age), woman's age at menopause and caucasian race. Despite the identification of many risk factors, in 75-80% of cases no risk factor is found(4). The risk factors can be divided in inherent and extrinsic. Inherent factors include sex, age, race, genetics and constitute independent parameters and do not undergo simple modification in the course of an individual's life(4). Extrinsic factors are conditioned by lifestyle, diet or long-term medical intervention and their influence on the neoplastic process may be modified to a certain degree(5). Deleterious mutations in the *BRCA1* and *BRCA2* genes are responsible for most hereditary breast cancers and are implicated in about 10-15% of these cancers(6).

A relevant obstacle to early identification of breast cancer is the development of accurate and convenient exams able to identify individuals potentially affected by the disease(7). Early detection of breast cancer allows easier treatment (minor surgery, less radiation or chemotherapy) and increased survival(8). Currently, screening with mammography is considered the gold standard for early breast cancer detection(9). However, the sensitivity of this exam varies between 54% and 77%, depending on the type of mammographic procedure(10). False positive rates in breast cancer

screening constitute an important limitation, causing unnecessary biopsies (11).

To confirm the diagnosis of breast cancer, breast biopsies such as core biopsy or mammotomy followed by histopathological and immunohistochemical analyzes are used, although limitations in these methods have already been reported(12). The biopsy procedure is invasive and in some cases associated with patient morbidity. The relative complexity, low access and high costs of the gold standard approach employed to diagnose the vast majority of breast cancer cases have prompted the search for alternative diagnostic methods and biomarkers to improve early detection(12).

According to the US Cancer Institute, a biomarker is a biological molecule found in blood, body fluids, saliva or tissues, representing a sign of a normal or abnormal process, or a sign of a disease condition such as cancer(13). Biomarkers typically differentiate a patient affected by a disease from a healthy individual. These changes can occur due to a number of factors including somatic or germline mutations, transcriptional changes, and post-translational changes.

There are a wide variety of biomarkers, including proteins, nucleic acids, antibodies, peptides, metabolites, among others(14). The biomarker definition of the Biomarker Working Group is: a cellular, biochemical and molecular alteration by which a simple, normal or abnormal biological process can be recognized or monitored and used to objectively measure and evaluate normal biological processes, pathogenic processes or pharmacological responses to therapeutic interventions(15).

A cancer biomarker refers to a substance or process that indicates the presence of cancer in the body. It may be a tumor-secreted molecule or it may be an organism-specific response to the presence of the neoplasm(13). Evidence suggests that serum and salivary biomarkers are viable options for diagnosis(14). Protein biomarkers can facilitate early detection of diseases at a stage that allows them to cure and can help distinguish subgroups of

patients who respond to certain types of treatments from those who do not(16).

Biomarkers may be used in patient follow-up, for example in the setting of metastatic disease(17). Circulating tumor protein soluble markers such as carcinoembryonic antigen (18), prostatic specific antigen (PSA), cancer antigen 125 (CA125), cancer antigen 15-3 (CA15-3) and cancer antigen 19-9 (CA19-9) antigens are respectively used to follow patients with colorectal, prostate, ovary, breast and pancreas cancer to assess response to treatment(19). Despite its widespread use, the only biomarker with high level of evidence for use in follow-up is the CEA for colorectal cancer(20). To date, the American Oncology Association, in its guidelines, does not recommend the use of biomarkers for the follow-up of breast cancer patients, and even the use of biomarkers for the diagnosis of the disease remains uncertain(21).

Recent technological advances in proteomics, metabolomics and transcriptomics have resulted in the identification and characterization of salivary components that may be useful for the diagnosis, prognosis and / or therapeutic follow-up of many diseases(22-26). Investigation of salivary biomarkers has developed beyond oral disease to systemic diseases (27, 28), expanding their detection potential (29-33). Saliva-based translational research and technology is now in a mature phase and can be evaluated to determine its usefulness in the detection of malignancies, since a sensitive assay readily identifies biomarkers using collected clinical specimens(30). A non-invasive method would be ideal for the detection and screening of malignant disease(26). As a diagnostic tool, saliva has several biochemical advantages compared to blood(34). Its collection is safe (ie without needle punctures), noninvasive and relatively simple. In addition, it can be collected repeatedly without discomfort for the patient(34).

Cancer antigen 15-3 (CA15-3) is a 300-400 kilodalton glycoprotein (KDa) produced by glandular epithelial cells (35). Serum levels of CA15-3 detect soluble forms of mucin 1 (MUC1), an aberrantly overexpressed transmembrane glycoprotein in breast cancers(36). The MUC1 protein is a broad glycosylated transmembrane molecule containing three major domains:

a large extracellular region, a sequential expansion membrane, and a cytoplasmic domain(37) (see Figure 1). Although the physiological function of MUC1 is unclear, this glycoprotein has been implicated in cell adhesion, lymphatic invasion and metastasis(38).

The first radioimmunometric assay for the identification of CA15-3 in the blood was performed in 1985 and was based on two monoclonal antibodies: DF3 and 115D8 (39). The most commonly used assays to detect serum CA15-3 are based on the 115D8 (used as a capture antibody), DF3 (as a detection antibody) sandwich-based Enzyme-Linked Immunosorbent Assay (ELISA) and chemiluminescence methods(40). The cutoff value for the serum CA15-3 level is set between 25 and 40 U/ mL(41).

Although many molecules have been investigated as presumed markers for breast cancer, only a few have sufficient sensitivity and specificity to be clinically useful. Studies indicate that serum CA15-3 sensitivity varies according to tumor mass and disease stage: 9% at stage I, 19% at stage II, 38% at stage III, and 75% at stage IV(42). In the initial phase, only 23% of cases have increased this marker and therefore, due to its low sensitivity and relatively low specificity for detection of early breast cancer, CA15-3 dosing is not recommended for screening, diagnosis or for evaluation of suspected breast lump(42). CA15-3 is widely used for the early diagnosis of relapse, preceding clinical signs by up to 13 months (43). Regarding prognosis, patients with preoperative values greater than 40 U/ mL have a 77% probability of relapse in 5 years(44). Only 1.3% of the healthy population has high concentration levels of serum CA15-3(45). Altered values may occur in pancreatic, lung, liver, ovarian, and cervical cancer, or, more rarely, in benign breast diseases and liver disease(46).

The presence of CA15-3 in saliva was discovered in the 2000s in women diagnosed with breast cancer(47). However, sufficiently sensitive and reproducible diagnostic methods based on saliva are not yet available. Due to the importance of CA15-3 in breast cancer patients that many times require needle puncture exams and have the risk of lymphedema, a search for a method with less morbidity such as CA15-3 in saliva is justified.

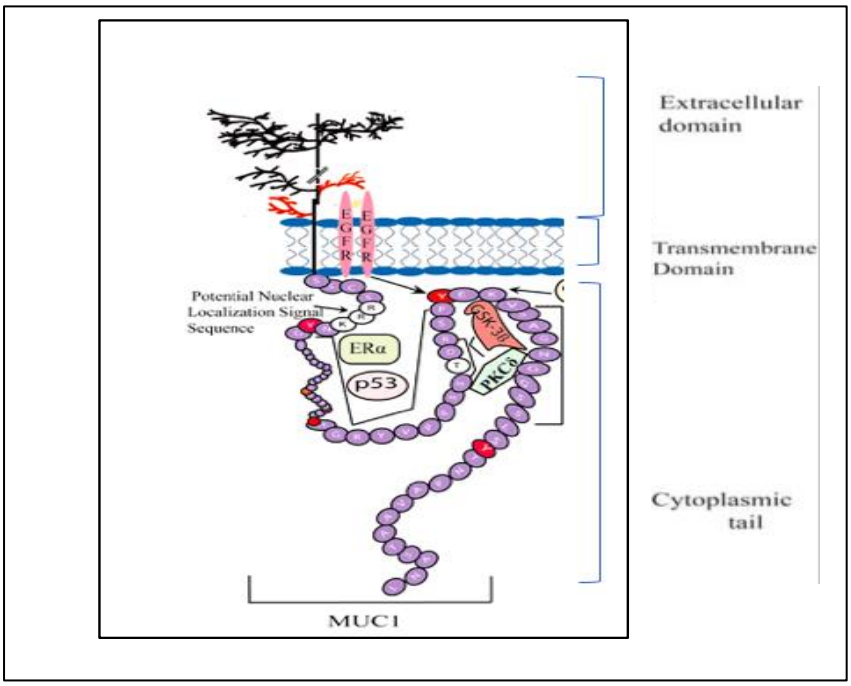


Figure 1. Schematic representation of the MUC1 protein. Withdrawn and adapted from Bafna *et al*.(48)

Currently, salivary biomarkers can be characterized or quantified by biochemical or immunohistochemical means and by genomic, proteomic, transcriptomic and through metabolomics, an emerging and promising method (26, 49-51). Saliva-based diagnostics, particularly those based on metabolomic technologies offer a clinical strategy that can characterize the association between salivary analysis and a particular disease(52).

Cancer metabolism is one of the oldest areas of research in cancer biology, predating the discovery of oncogenes and tumor suppressors by some 50 years(53). The field is based on the principle that metabolic activities are altered in cancer cells relative to normal cells, and that these alterations support the acquisition and maintenance of malignant properties(53). Most of the classical examples of reprogrammed activities either support cell survival under stressful conditions or allow cells to grow and proliferate at pathologically elevated levels(54).

Metabolic alteration is a hallmark of cancer cells and malignant transformation is characterized by the occurrence of multiple changes in metabolic pathways that are linked to the synthesis of macromolecules (55,

56). Figure 2 ilustrates the hypothetical links between different metabolic alterations and the seven Hallmarks of Cancer. Cancer cells use the similar metabolic network to that of the normal tissues from which they originated and have altered metabolism in order to proliferate and survive in an adverse environment(57). Cancer phenotypes are associated to aerobic glycolysis, de novo lipid biosynthesis and glutamine-dependent anaplerosis among other metabolic alterations and can be caused by metabolic gene expression after activation of growth signaling of normal and malignant cells(58).

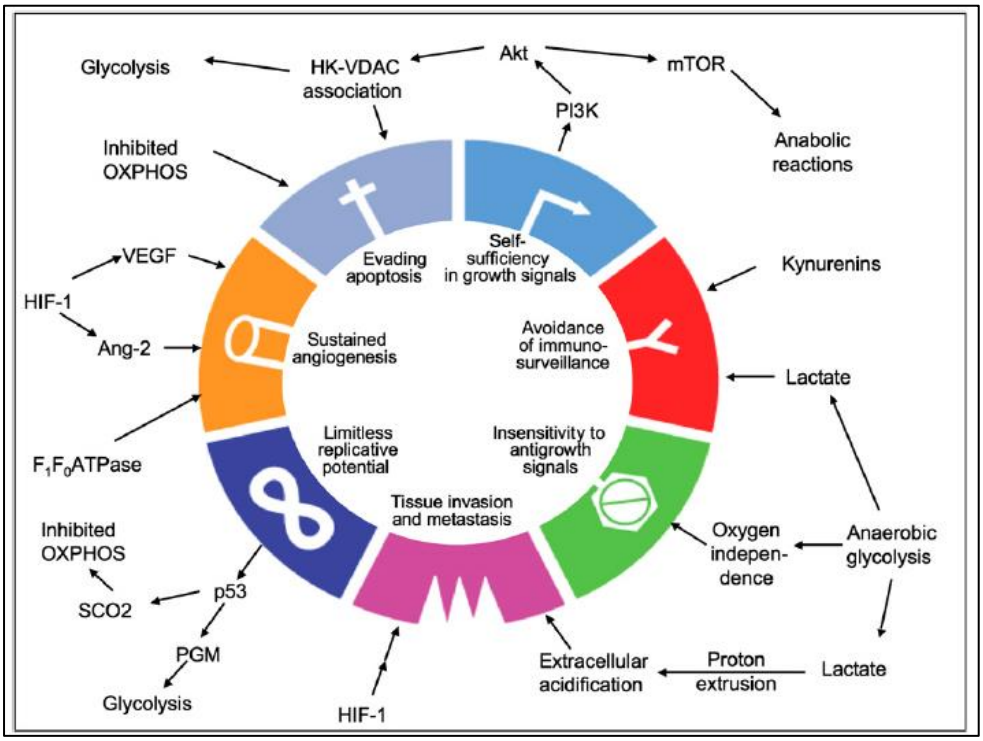


Figure 2. The links to tumor metabolism and the seven Hallmarks of Cancer (evading apoptosis, self-sufficiency in growth signals, avoidance of immune surveillance, insensitivity to antigrowth signals, tissue invasion and metastasis, limitless replicative potential and sustained angiogenesis). Centripetal arrows (pointing from the inside outwards) indicate how the seven hallmarks of cancer can impinge on metabolism. Centrifugal arrows (pointing from the outside inwards) illustrate how neoplasia-associated metabolic reprogramming can contribute to the acquisition of the seven hallmarks. Ang-2, angiopoietin-2; GLUT, glucose transporter; HIF, hypoxia-inducible factor; HK, hexokinase; OXPHOS, oxidative phosphorylation; PGM, phosphoglycerate mutase; PI3K, phosphatidylinositol 3-kinase; SCO2, synthesis of cytochrome c oxidase 2; VDAC, voltage-dependent anion channel; VEGF, vascular endothelial growth factor. From Kroemer *et al.*(59)

The classical example of a reprogrammed metabolic pathway in cancer is the Warburg effect or aerobic glycolysis(60). Glycolysis is a physiological response to hypoxia in normal tissues, but Otto Warburg in the 1920s observed that tumor slices and ascites cancer cells constitutively take up glucose and produce lactate regardless of oxygen availability, an observation that has been seen in many types of cancer cells and tumors(61). Figure 3 represents a scheme of aerobic glycolysis. Otto Warburg's hypothesis that cancer cells take up glucose and generate a substantial amount of lactate in the presence of ambient oxygen due to impaired mitochondrial function led to the widely held misconception that cancer cells rely on glycolysis as their major source of adenosine triphosphate (ATP) (62). Today, it is clear that cancer cells exhibit aerobic glycolysis due to activation of oncogenes, loss of tumor suppressors, and up-regulation of the phophoinositide-3-kinase (PI3K) pathway, and that one advantage of high glycolytic rates is the availability of precursors for anabolic pathways(53).

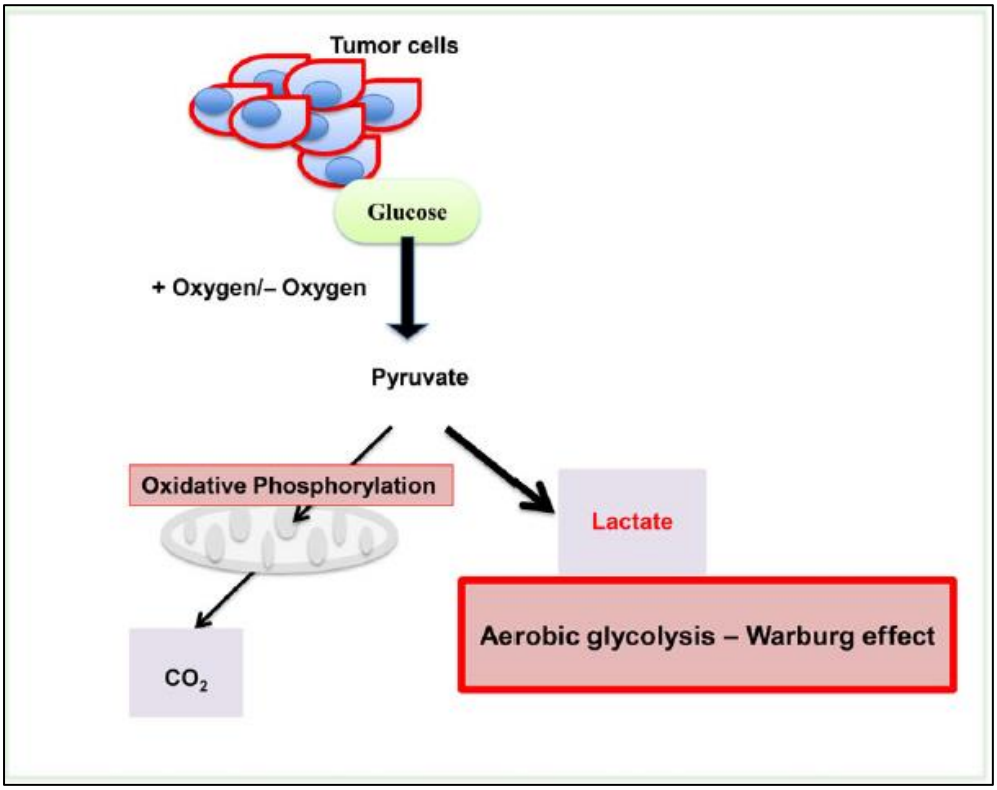


Figure 3. Representation of the aerobic glycolysis phenomenon in cancer cells. Tumor cells can convert most of the available glucose to produce lactate regardless of the presence or absence of oxygen. This phenomenon in called Warburg effect, where cancer

cells showed a high rate of glycolysis and a decrease in mitochondrial respiration. From Ray *et al.*(63)

The increase in glycolytic flux allows glycolytic intermediates to supply subsidiary pathways to fulfill the metabolic demands of proliferating cells(60). Like glycolytic intermediates, tricarboxylic acid cycle intermediates are also used as precursors for macromolecule synthesis(6). Their utilization in biosynthetic pathways requires that carbon be resupplied to the cycle so that intermediate pools are maintained; pathways that “refill” the cycle are termed anaplerotic pathways, and they genarate tricarboxylic acid (6) cycle intermediates that can enter the cycle at sites other than acetyl-coenzyme A (CoA) (6, 53).Two activities that provide anaplerotic fluxes in cancer cells are glutaminolysis, which produces a-ketoglutarate from glutamine, and pyruvate carboxylation, which produces oxaloacetate from glucose/pyruvate(64). Oxidation of the branched-chain amino acids (BCAAs) isoleucine and valine also provides an anaplerotic flux in some tissues(53).

In addition to pyruvate derived from glycolysis, fatty acids and amino acids (AA) can supply substrates to the TCA cycle to sustain mitochondrial ATP production in cancer cells(65). The breakdown of fatty acids (β-oxidation) in the mitochondria generates acetyl-CoA and the reducing equivalents NADH and FADH2, which are used by the electron transport chain (ETC) to produce mitochondrial ATP(66). The amino acid glutamine can generate glutamate and subsequently a-ketoglutarate to fuel the TCA cycle through a series of biochemical reactions termed glutaminolysis(67). Furthermore, the AAs isoleucine, valine, and leucine, which are elevated in plasma of patients with cancers, can be converted into acetyl-CoA and other organic molecules that also enter the TCA cycle(68). The metabolic flexibility afforded by multiple inputs into the TCA cycle allows cancer cells to adequately respond to the fuels available in the changing microenvironment during the evolution of the tumor(53). Figure 4 shows a schematic representation of aerobic glycolysis and oxidative phosphorylation.

Metabolomics describes the study of concentrations and fluxes of low molecular weight (MW) metabolites present in biofluids or tissue that provide

detailed information on biological systems and their current status(69). The principal analytical tools recruited for metabolome analysis are mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR)(70). MS can be coupled with a separation technique such as gas chromatography (GC-MS), liquid chromatography (LC-MS) or capillary electrophoresis (CE-MS)(51).

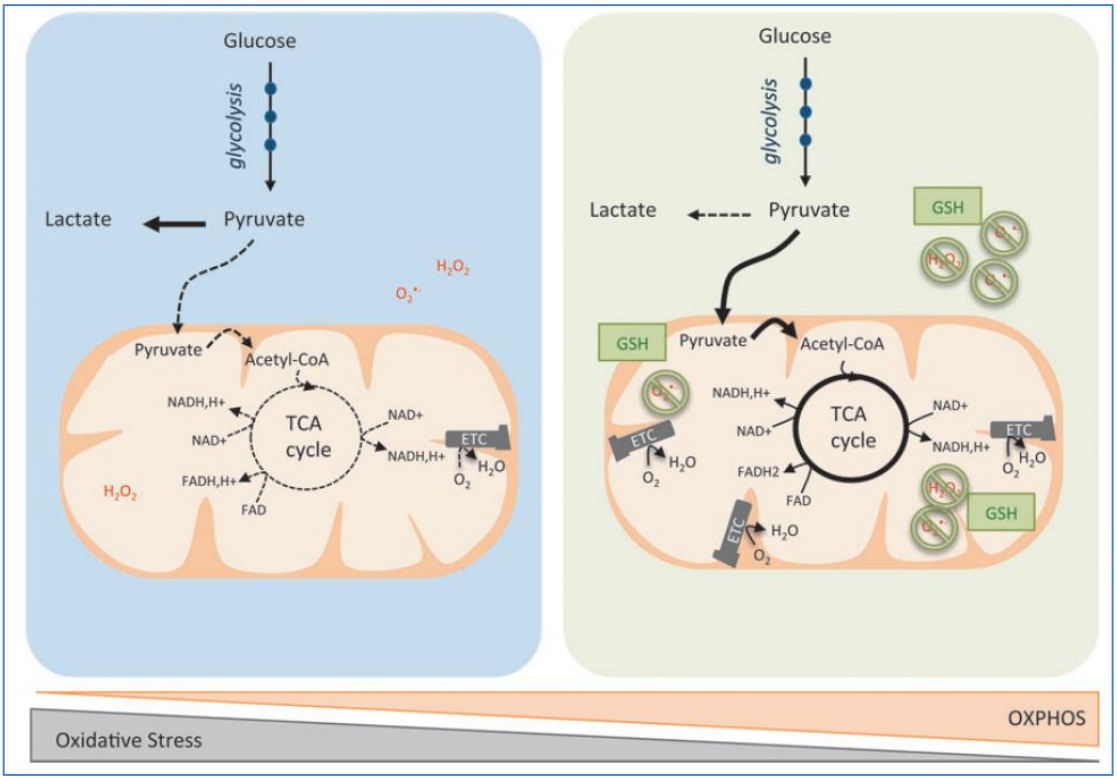


Figure 4. Schematic representation of aerobic glycolysis and oxidative phosphorylation. Blue left panel is a schematic representation of cancer cells relying predominantly on aerobic glycolysis. Pyruvate is preferentially oxidized into lactate (dark line). Consequently, acetyl-CoA is less incorporated into the TCA cycle (dashed line), which leads to decreased production of reducing equivalents. Some cancer cells exhibit a reciprocal phenotype, with enhancement of the OXPHOS metabolism (green right panel). Here, pyruvate is oxidized into acetyl-CoA and subsequently metabolized into the TCA cycle (dark lines), but less converted into lactate (dashed line). Mitochondrial respiration produces ATP and oxidizes electrons from reduced cofactors and reduces O2 into H2O through the ETC complexes. The various single-electron intermediates can escape and react with O2 forming ROS. OXPHOS cancer cells show elevated antioxidant programs, which help them to detoxify ROS produced by the ETC and regenerate reduced GSH. GSH, glutathione; H2O2, hydrogen peroxide; H2O, water; OXPHOS, oxidative phosphorylation; O2, oxygen; $O2^{(-)}$, superoxide anion radical; ROS, reactive oxygen species. From Gentric *et al*. (71)

The metabolomics experiments can have two designs: targeted and untargeted. In untargeted approach, metabolites are first isolated from tissues, biofluids, or cell cultures and subsequently analyzed by LC/MS(72). After data acquisition, the results are processed by using bioinformatic software such as XCMS to perform nonlinear retention time alignment and identify metabolite features that are changing between the groups of samples measured(73). Metabolite features of interest are searched in metabolite databases on the basis of accurate mass to obtain putative identifications. Putative identifications are then confirmed by comparison of MS/MS and retention time data to that of standards(74). The untargeted workflow is global in scope and outputs data related to comprehensive cellular metabolism(73).

In targeted metabolomic workflow, standard compounds for the metabolites of interest are obtained and used to setup selected reaction monitoring methods. Instrument voltages are established and concentration curves are generated for absolute quantitation(75). After the targeted methods have been established on the basis of standards, the metabolic extract is analyzed from the research samples(76). The data output provides quantitation only of those metabolites for which standard methods have been built(73). Figure 5 ilustrates the untargeted and targeted flows for LC/MS based metabolomics.

Differences in serum and salivary metabolites of cancer patients and healthy controls have already been described(77, 78). Combination serum of metabolites may be a good marker for oral cancer (OC). Propionate + acetone + acetate + choline had good diagnostic value with sensitivity of 90.9% and specificity of 96.0%. The metabolites not only discriminates control and disease samples but also exhibits noteworthy potential to differentiate oral leukoplakia and oral cancer stages of disease-sample with high specificity(78). Salivary choline + betaine + pipecolinic acid+ L-carnitine give a predictive value with 100% sensitivity and 96.7% specificity for OC diagnosis(79). The combination of salivary L-phenylalanine and L-leucine revealed sensitivity (92.3%) and specificity (91.7%) for early diagnosis of OC(80). Salivary propionylcholine, N-acetyl-L-phenylalanine, sphinganine, phytosphingosine, and S-carboxymethyl- L-cysteine in combination yielded sensitivity 100% and

specificity 96.7% in distinguishing early stage of OC(25). Eighteen potential metabolites for diagnosing breast cancer (BC) were identified and three up-regulated metabolites, LysoPC (18:1), LysoPC (22:6) and MG (0:0/14:0/0:0) provided area under curve (AUC) values of 0.920, 0.920 and 0.929 respectively, showing a high accuracy in predicting BC(81).

Given the growing interest in salivary diagnosis, different authors emphasize the need for standardization of sample collection and the development of multi-marker detection tools validated for specificity and sensitivity(82). These findings justify the study and research of salivary biomarkers for the diagnosis of chronic diseases such as breast cancer, that is the most common type of cancer and the leading cause of cancer death among women worldwide, excluding non melanoma skin cancer(1).

Due to all these important features, we will study the presence of biomarkers in saliva such as CA15-3 and metabolites of breast cancer patients and healthy controls, in order to evaluate their diagnostic capacity. Since saliva is easily accessible, it becomes an attractive test fluid especially for cancer patients, who routinely and systematically undergo invasive tests with various drawbacks.

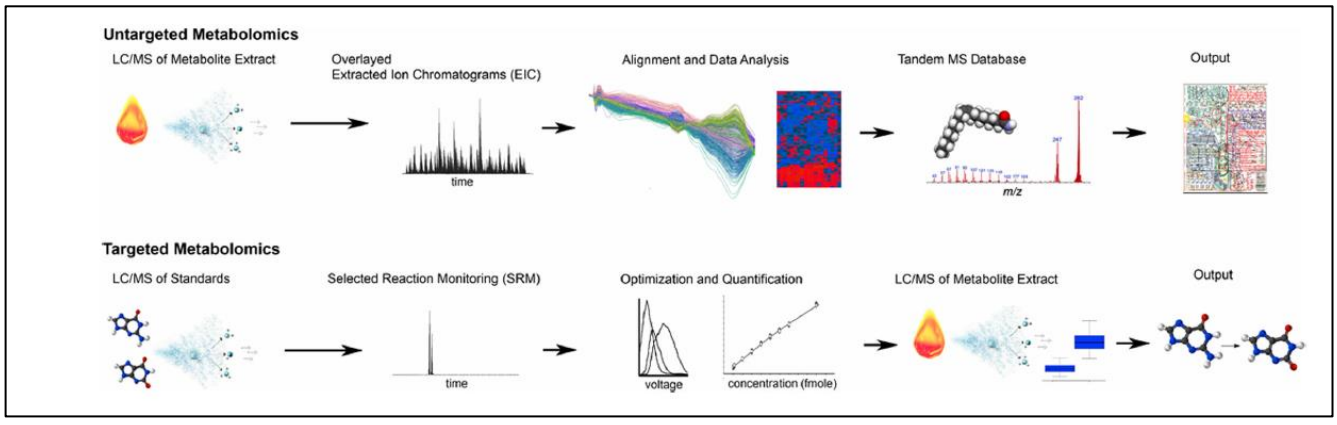


Figure 5. Untargeted and targeted workflow for LC/MS metabolomics. From Patti *et al*. (83) The untargeted metabolomic workflow: metabolites are first isolated and subsequently analyzed by LC/MS. After data acquisition, the results are processed by using bioinformatic software such as XCMS to perform nonlinear retention time alignment and identify metabolite features that are changing between the groups of samples measured. Metabolite features of interest are searched in metabolite databases on the basis of accurate mass to obtain putative identifications.

2 ARTICLES

2.1 Article 1: Correlation between salivary and serum CA15-3 concentration in breast cancer patients

Daniele Xavier Assad[1,2], Elisa Cançado Porto Mascarenhas[1,3], Ana Gabriela Costa Normando[1], Helene Chardin[4,5], Gustavo Barcelos Barra[6], Riccardo Pratesi [7], Yanna Karla de Medeiros Nóbrega[8], Ana Carolina Acevedo[1], Eliete Neves Silva Guerra[1*]

[1] Laboratory of Oral Histopathology, Health Sciences Faculty, University of Brasília

Campus Universitário Darcy Ribeiro, Brasília DF, Brazil

[2] Medical Oncology Department, Hospital Sírio-Libanês, SGAS 613 Conj. E Bl. B, Brasília DF, Brazil

[3] Medical Oncology Department, Cettro- Centro de Câncer de Brasília, SMH/N Quadra 02, 12º Andar, Brasilia, DF, Brazil.

[4] Department of Analytical, Bioanalytical Sciences and Miniaturization (LSABM), ESPCI Paris, UMR CBI 8231, PSL Research University, Paris, France

[5] Faculté de Chirurgie Dentaire, Université Paris Descartes Sorbonne Paris Cité, Paris, France

[6] Sabin Medicina Diagnóstica, SAAN Quadra 03 lotes 145/185, 70632-340, Brasília – DF, Brazil

[7] Interdisciplinary Laboratory of Biosciences and Celiac Disease Research Center, School of Medicine, Campus Universitário Darcy Ribeiro, University of Brasilia, Brasilia, Brazil

[8] Applied Analysis Laboratory, Department of Pharmaceutical Sciences, Health Sciences Faculty, University of Brasília, Campus Universitário Darcy Ribeiro, Brasília DF, Brazil

Abstract

Early detection of breast cancer enables the use of less aggressive treatment and increases the chance of survival. The transmembrane glycoprotein mucin 1 (MUC1), also known as cancer antigen 15-3 (CA15-3), is aberrantly glycosylated and overexpressed in a variety of epithelial cancers, and plays a crucial role in progression of the disease. In the present study, CA15-3 concentrations in saliva and blood of patients with breast cancer were evaluated. There are no previous reports of the use of chemiluminescence assay (CLIA) and electrochemiluminescence assay (ECLIA) in saliva. Saliva and blood were collected on the same day from breast cancer patients (N=26) and healthy controls (N=28). For each individual subject, the level of serum CA15-3 was measured by ECLIA, and the level of salivary CA15-3 was measured by ECLIA, CLIA, and enzyme-linked immunosorbent assay (ELISA). ELISA and CLIA were able to detect CA15-3 in saliva, however ECLIA could not detect salivary CA15-3. There was no significant difference between mean serum and salivary CA15-3 levels in breast cancer patients or healthy controls ($p=0.41$). However, the mean values for CA15-3 in serum were higher in breast cancer patients than in healthy controls. The levels of CA15-3 were highest for luminal breast cancer subtypes and stage IV cases. There was a moderate correlation between salivary and serum CA15-3 levels as measured by ELISA in breast cancer patients ($r=0.56$, $p=0.0047$). The results showed that ECLIA was not a good method to detect salivary CA15-3, although it is the golden standard for detecting serum CA15-3. The presence of CA15-3 in saliva was confirmed and this will be useful in future research. Further investigations are necessary to confirm the capability of detection of salivary CA15-3 and its correlation to serum CA15-3.

Keywords: breast cancer, saliva, CA15-3, electrochemiluminescence, enzyme-linked immunosorbent assay, chemiluminescence.

1 INTRODUCTION

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death among females (1). A multidisciplinary approach involving surgical, radiation, and systemic treatments has contributed to a reduction in breast cancer mortality in recent years (2). The decrease in mortality is likely attributable, in part, to improved breast cancer screening and adjuvant therapy (3).

The early diagnosis of breast cancer is vital to increase survival rates, decrease morbidity, and reduce the likelihood of recurrence of disease (4). Breast cancer diagnosed at an early stage is more likely to be treated successfully and has a better prognosis. When the initial tumor burden is advanced, the patient's chances of survival are much lower (5).

Breast cancer diagnosis has two main steps: the identification of a suspected lesion with radiological screening examinations, and a confirmatory biopsy (2). Conventional screening with physical examination and mammography has less-than-desirable sensitivity (54%) and specificity (77%) [6]. Breast biopsy and histopathology studies are the reference standard for diagnosis; however, they have limitations owing to the invasiveness of the procedure and the risk of morbidity (7). In this context, emerging research has focused on breast cancer biomarkers as a potential adjunctive diagnostic tool.

Members of the human mucin (MUC) family — designated MUC1 to MUC21 — are cell surface receptors and have been sub-classified into secreted and transmembrane forms (8). MUC1 is a transmembrane member of the mucin family that is aberrantly glycosylated and overexpressed in a variety of epithelial cancers after transformation and loss of polarity (9). MUC1 localizes in the apical membranes of normal secretory epithelial cells and provides protection to the underlying epithelia in healthy tissues, maintaining homeostasis and, therefore, promoting cell survival in variable conditions (10). Tumor-associated MUC1 differs from the MUC1 expressed in normal cells and participates in intracellular signal transduction pathways and regulates

the expression of its target genes at both the transcriptional and posttranscriptional levels (11).

Cancer antigen 15-3 (CA15-3) is a soluble form of the transmembrane glycoprotein mucin 1 (MUC1). CA15-3 corresponds to an immunodominant epitope in the extracellular portion of the protein that is shed into the bloodstream and can be detected by several monoclonal antibodies (12). CA15-3 is the most widely used serum marker to detect recurrent breast cancer and monitor treatment of patients with advanced disease (13).

Human saliva mirrors the body's health and most of the biomolecules that are present in blood or urine can also be found in salivary secretions (14). It offers several benefits over traditional blood-based biochemical analyses for clinical diagnostics: non-invasiveness and stress-free sample collection; easy and multiple sampling opportunities; reduced need for sample pre-processing; minimal risk of contracting infectious organisms such as human papilloma virus, hepatitis B, and human immunodeficiency virus (15). In the last decade, saliva has emerged as a source of biochemical data to detect chronic diseases, as it may contain real-time information describing the overall physiological condition (84).

Enzyme-linked immunosorbent assay (85), electrochemiluminescence (86), and chemiluminescence (CLIA) are the most frequently used methods to assess serum levels of CA15-3 (17-21). Several methods for evaluating salivary levels of CA15-3 have been used, however, the most frequently reported is ELISA (19, 20, 22-24). There are no reports of the application of CLIA and ECLIA to detect CA15-3 salivary levels.

Owing to the worldwide importance of breast cancer and the need to evaluate alternative methods for detecting salivary levels of CA15-3, such as ECLIA and CLIA, the aim of this study was to evaluate ELISA, CLIA, and ECLIA methods for quantifying the levels of CA15-3 in saliva and serum of breast cancer patients compared to healthy controls. We found that salivary CA15-3 could be detected using ELISA and CLIA, but not ECLIA.

2 PATIENTS AND METHODS

Subjects

Subject recruitment and sample collection followed the guidelines of the Institutional Review Board of the oncology recruiting centers: Hospital Universitário de Brasília (HUB), Hospital de Base do Distrito Federal (HBDF), Hospital Sírio Libanês and Centro de Câncer de Brasília- Cettro. The cohort study was approved by the Research Ethics Committee of the Faculty of Health Sciences at the University of Brasilia (UnB-DF, Brazil) through Plataforma Brasil protocol 57449716.5.0000.0030, and was conducted according to the Declaration of Helsinki principles. Written informed consent was obtained from each subject before participation in the study.

The inclusion criteria for the breast cancer patients group were as follows: i) capable of giving informed consent; ii) not pregnant or lactating; iii) no active oral/dental disease; iv) no prior neoplasia (except for non-melanomatous skin cancers and carcinoma in situ of the cervix, or benign tumors such as adenomas), and no alterations of renal function, congestive heart failure, active infection hepatitis or HIV; and v) a proven histopathologic diagnosis of breast cancer. These patients were enrolled prior to definitive surgery for the excision of the primary tumor and prior to systemic treatment (neo-adjuvant chemotherapy or palliative endocrine/ or chemotherapy). All patients were recruited by convenience after appointment at an oncology center. The control subjects were healthy female volunteers recruited from the general population, for whom breast cancer was ruled out by physical examination and radiological breast images. None of the participants in the control group were knowingly suffering or being treated for a malignancy.

Specimen collection, transportation, and preparation

Venous blood and saliva samples were collected on the same day for each participant. All participants abstained from eating, drinking, smoking and performing oral hygiene procedures for at least 1 hour prior to collection of

saliva. For saliva collection, participants were instructed to chew on a cotton swab (Salivette®, Sarstedt AG & Co, Nümbrecht, Oberbergischer Kreis, Germany) for a period of 2 minutes. Each swab containing saliva was returned to a separate plastic container and then packaged in a styrofoam with recyclable ice sheets. Within 4 hours, the material was transported to the laboratory for processing. The saliva sample was centrifuged for 5 minutes at 3,000 rotations per minute (rpm) at 8 °C. After centrifugation, the sample was transferred to a clean Eppendorf tube and frozen at -80 °C until processing. The saliva samples were thawed at room temperature for CA15-3 analysis. Typically, patients donated 5–10 mL of saliva.

Blood samples were obtained by venipuncture and were collected in serum tubes with separator gel. Blood was centrifuged at 3,500–5,000 rpm for 5 minutes and the total volume obtained was separated into 2 Eppendorf tubes and frozen at -20 °C until samples were analyzed.

Electrochemiluminescence assay (ECLIA) for detection of serum and salivary CA15-3

Measurement of serum CA15-3 was performed by ECLIA on a fully automated Roche Cobas 8000 analyzer with an e801 module (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions and reported in U/mL. The development of ECLIA is based on the use of a ruthenium-complex and tripropylamine (TPA). The chemiluminescence reaction for the detection of the reaction complex is initiated by applying a voltage to the sample solution resulting in a precisely controlled reaction. The limit of blank, limit of detection, and limit of quantification for measuring CA15-3 in serum with the Cobas e801 module are 1.0 U/mL, 1.5 U/mL, and 3 U/mL, respectively (Elecsys CA15-3 II Label, 07027001500V2.0) (11).

Measurement of salivary CA15-3 was performed as described above for serum; however, saliva is an off-label specimen for the applied assay.

Chemiluminescence assay (CLIA) for detection of salivary CA15-3

Measurement of salivary CA15-3 was performed according to the manufacturer's instructions using a sandwich CLIA with the BR-MA 15-3

reagent kit in an IMMULITE 1000® system (Siemens Healthcare Diagnostics Inc., Erlangen, Germany). The kit for serum assay was used for salivary assay; however, saliva is an off-label specimen for the applied assay.

The microtiter plates were pre-coated with an antibody specific for the analyte. Standards or samples were added to the appropriate microtiter plate wells, where the analyte present in the standards and samples would bind to the immobilized antibody. Next, biotin-conjugated antibody was added and bound to the analyte on the plate. The complex of two antibodies and the analyte in the wells forms a “sandwich” structure. After any unbound biotin-conjugated antibody was removed by washing, avidin-conjugated horseradish peroxidase was added to each microplate well. After incubation at 25^{o}C for 20 minutes, luminol was added into the wells. Relative luminescence intensity was determined using a photomultiplier, in relative light units (RLU), being proportional to the amount of CA15-3 present in the sample, and the results were expressed as U/mL. The assay limit of detection for CA15-3 is 1.0 U/mL (25

Enzyme-linked immunosorbent assay *(*ELISA) assay for detection of salivary CA15-3

ELISA reactions were performed using the CA15-3 AccuBind ™ reagent kit (Lake Forest, California, United States of America) for use in BEST 2000® equipment (Biokit, Barcelona, Spain), according to the manufacturer’s instructions. The kit used for salivary assay was the same as that used for serum assay; however, saliva is an off-label specimen for the applied assay. The assays are a two-site solid phase enzyme immunoassay. The molecules of the antigens of interest are “sandwiched” between two monoclonal antibodies, the first one attached to the ELISA solid phase and the second one linked to the horseradish peroxidase (enzymatic conjugate). After washing, the enzymatic reaction develops a color proportional to the quantity of CA15-3 antigen present. The absorbance was read at 450nm using a spectrophotometer and concentrations were calculated from standard curves constructed from known concentrations of the ligand.

For the calculation of the results, a standard-logarithmic curve was obtained by plotting the measured values of the 6 calibrators by the corresponding units (linear/log). The analysis was performed in duplicate, and the mean of the two values obtained was calculated. The results were expressed as U/mL.

For this assay, the limit of blank and functional limit of detection for measuring CA15-3 in serum are 0.2 U/mL and 1.25 U/mL, respectively (AccuBind ™ reagent kit, Revision: 3, Date: 072611, Cat #: 5625-300, DCO:0504)(25).

TNM and molecular profile of breast cancer

TNM staging was performed according to the 7th edition of the AJCC (26), and the molecular profile classification was determined in accordance with the immunohistochemical definitions of the Saint Gallen consensus (27). The median of the levels of serum and salivary CA15-3, detected by ECLIA, CLIA, and ELISA, were determined for each patient based on TNM and molecular profile.

Statistical analysis

Statistical analysis was made with SAS 9.4 version 9.4. Student's t test and chi-square/Fisher exact test were applied to demographic and clinical characteristics. A Mann–Whitney U test was used to compare mean values of serum and salivary CA15-3 among controls and breast cancer patients. A Kruskal–Wallis test was used to compare mean values of salivary CA15-3 and serum CA15-3 among molecular subtypes and stages and, when $p<0.05$, multiple comparisons were implemented using the Dwass, Steel, Critchlow-Fligner (DSCF) method. Correlations of serum and salivary CA15-3 in controls and breast cancer patients were assessed using the Pearson correlation coefficient. Values of $p \leq 0.05$ were considered statistically significant.

3 RESULTS

Table 1 summarizes the characteristics of the 28 control subjects and 26 breast cancer patients. The mean age of the controls was lower than of

the breast cancer patients (37.64+/-13.57 years *versus* 48.23+/-11.51 years, p=0.0033). There were more postmenopausal women among breast cancer patients than in the controls (11 *versus* 4, p=0.0216). There was no significant difference between the healthy controls and cancer patients regarding tobacco use, medication use, and presence of systemic disorders (p=0.1842, p=0.5541, and p=0.8473, respectively). Mean body mass index was significantly higher in breast cancer patients than in controls (p=0.0184).

Among the 26 breast cancer patients, there were two stage I (7%), ten (39%) stage IIa, three (12%) stage IIb, four (15%) IIIb, one (3%) stage IIIc and five (23%) stage IV breast cancer cases. There were three (11.5%) luminal B-like HER2 negative, seven (27%) luminal A-like, five (19%) HER2 positive (nonluminal), four (15%) luminal B-like HER2 positive, and five (19%) triple negative (TN) breast cancer cases. There was no information for TNM in one patient and for the molecular profile in two patients. The complete information of the subjects is listed in Supplementary Table 1.

Table 1. Demographic data based on participants records.

	Groups		
Characteristics*	**Healthy Control (n = 28)**	**Breast Cancer (n = 26)**	**p-value#**
Age	37.64±13.57	48.23±11.51	0.0033
Body Mass Index	22.93 ± 3.14	25.39 ± 4.25	0.0184
Menopause status			0.0216
Premenopause	24 (85.71)	15 (57.69)	
Menopause	4 (14.29)	11 (42.31)	
Tobacco use			0.1842
No	27 (96.43)	19 (84.62)	
Yes	1 (3.57)	4 (15.38)	
Use of medication			0.5541

No	15 (53.57)	16 (61.54)	
Yes	13 (46.43)	10 (38.46)	
Systemic Disease			0.8473
No	19 (67.86)	17 (65.38)	
Yes	9 (32.14)	9 (34.62)	

* mean values ±standard deviation or frequence (%); # Student's t test and Qui-square/Fisher exact test

Serum (ECLIA) and salivary (CLIA and ELISA) values of CA15-3 for each subject are listed in S2 Table. Mean serum CA15-3 in breast cancer patients by ECLIA was 134 ± 369.00 U/ml and 15.7 ± 6.1 U/ml in healthy controls. Mean salivary CA15-3 in breast cancer patients by CLIA was 4.7 ± 5.70 U/ml and 6.5 ± 7.1 U/ml in healthy controls. Mean salivary CA15-3 measured by ELISA was 1.78 ± 1.0 U/ml in breast cancer patients and 1.83 ± 2.0 U/ml in healthy controls. Either the ECLIA assay was not able detect the CA15-3 protein in saliva or the CA15-3 levels in saliva were below the ECLIA assay limit of detection of 1.5 U/mL. The CLIA and ELISA limits of detection were 1.0 U/mL and 1.25 U/mL, respectively, hence ECLIA was the least sensitive among the tested assays. There was no significant difference between serum CA15-3 levels in breast cancer patients *versus* healthy women (p=0.0571), and there was no difference in salivary CA15-3 concentration between breast cancer cases and controls when measured by CLIA (p=0.1861) and ELISA (p=0.5554).

Table 2. Serum and salivary CA15-3 concentration for healthy controls and breast cancer patients

CA15-3	Healthy Control* (n = 28)	Breast Cancer* (n = 26)	p-value#
ECLIA Serum (U/mL)	15.73 ± 6.18	133.97 ± 369.02	0.0571
CLIA Salivary (U/mL)	6.51 ± 7.18	4.73 ± 5.74	0.1861
ELISA Salivary (U/mL)	1.83 ± 2.09	1.77 ± 1.08	0.5554

* mean values CA15-3 ±standard deviation ; # Mann-Whitney test

The CA15-3 concentrations in saliva and serum according to breast cancer molecular subtypes are listed in Table 3. The analysis was performed in 24 patients with a known molecular profile. There was no difference in mean concentration values for serum CA15-3 measured by ECLIA (p=0.20), salivary CA15-3 measured by ELISA (p=0.70) and CLIA (p=0.78) according to molecular subtype. ECLIA for serum CA15-3 revealed the highest values for luminal A subtype, with 269.47±659.97 U/mL CA15-3 concentration. The highest values for CA15-3 mean concentration in luminal B HER2+ subtype were 2.58±1.83 U/mL with ELISA and 10.57±11.74 U/mL with CLIA.

Table 3. CA15-3 mean concentration values + SD versus molecular subtypes

CA15-3	Luminal A*	Luminal B HER2 +*	Luminal B HER2 -*	HER2 positive*	Triple negative*	p-value#
ECLIA Serum (U/mL)	269.47±659.97	141.60±183.22	196.90±186.53	18.04±7.98	14.98±6.87	0.2040
ELISA Salivary (U/mL)	1.71±1.11	2.58±1.83	1.61±0.33	1.97±1.27	1.28±0.26	0.7069
CLIA Salivary (U/mL)	4.13±5.25	10.57±11.74	3.43±3.03	2.72±1.14	5.65±5.44	0.7823

ECLIA: electrochemiluminescence assay, CLIA: chemiluminescence assay, ELISA: Enzyme-linked immunosorbent assay * mean values± standard deviation # Kruskal-Wallis test

The CA15-3 mean concentrations in saliva and serum according to TNM stages are listed in Table 4. The analysis was performed in 25 patients with known TNM stage. There was no difference in salivary CA15-3 by ELISA (p=0.44) and CLIA (p=0.40) among different breast cancer stages. Serum CA 15-3 levels were significantly different in at least two stages of breast cancer (p=0.010). The DSCF multiple comparison test revealed differences in serum CA 15-3 concentrations among stage IV and stage IIa cases. The mean serum CA15-3 value for stage IV cases (508.20±718.32 U/mL) was higher than that for stage IIa (17.18±9.14 U/mL) (p=0.03). There were no significant differences for the other comparisons of mean serum CA15-3 values between stages. In all analyses in both serum and saliva, the TNM stage IV disease cases showed the highest mean CA15-3 concentration: 508.20±718.32 U/mL with ECLIA in serum, 2.73±1.82 U/mL with ELISA in saliva, and 7.78±9.70 U/mL with CLIA in saliva.

Among breast cancer cases, there was a significant positive correlation of serum CA15-3 and salivary CA15-3 with ELISA (r=0.56; p=0.0047); however no significant correlation of salivary CA15-3 and serum CA15-3 was observed with CLIA (p=0.19) Among healthy controls, the correlations of

salivary CA15-3 with serum CA15-3 with CLIA and ELISA were not significant (p= 0.77 and p=0.35 respectively). All correlations are shown in Fig 1.

Table 4. Mean CA 15-3 concentration values + SD versus Stage.

CA15-3	I*	II a*	II b*	IIIb*	IV*	p-value#
ECLIA& Serum (U/mL)	16.50±11.31	17.18±9.14	15.00±5.43	97.13±100.13	508.20±718.32	0.0129
ELISA Salivary (U/mL)	1.41±0.19	1.77±0.98	1.46±0.22	1.28±0.13	2.73±1.82	0.4458
CLIA Salivary (U/mL)	-	4.00±3.18	3.50±1.56	1.25±0.21	7.78±9.70	0.4035

ECLIA: electrochemiluminescence assay, CLIA: chemiluminescence assay, ELISA: Enzyme-linked immunosorbent assay

* mean values±standard deviation, # Kruskal-Wallis test, & - multiple comparisons test of Dwass, Steel, Critchlow-Fligner (DSCF). Comparison of stages IIa and IV (p = 0.0338)

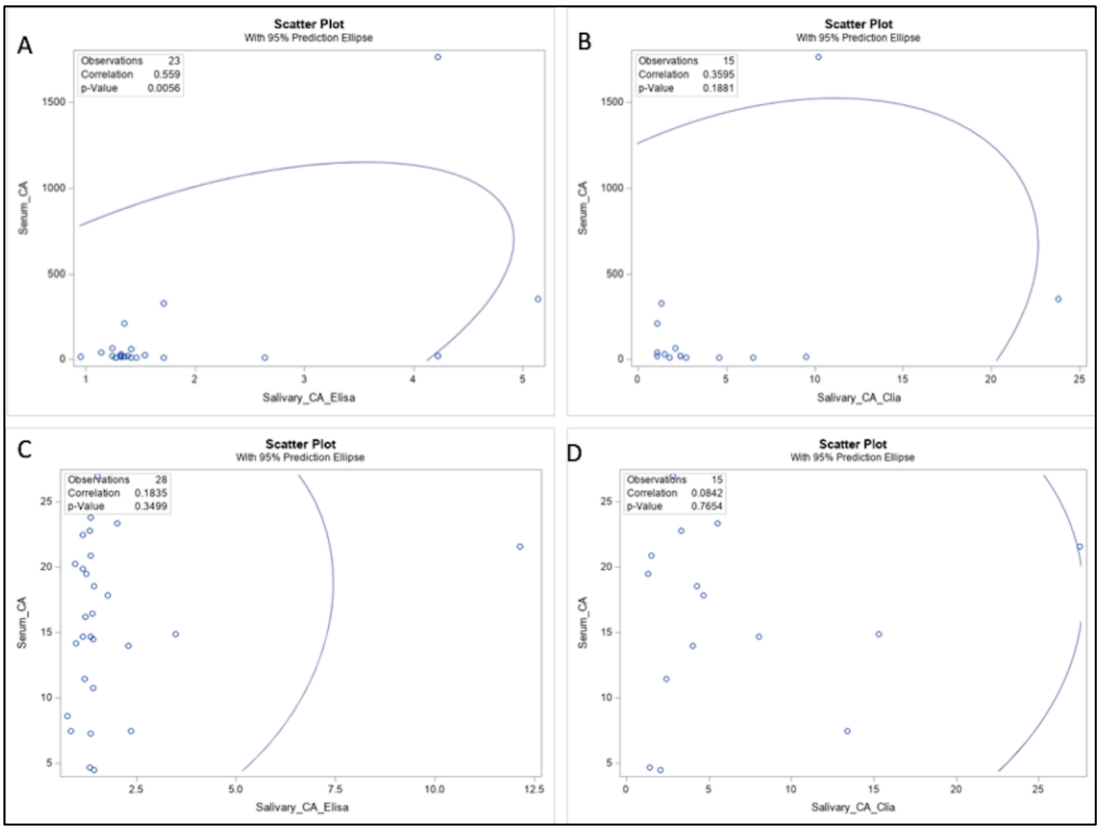


Fig 1. Correlations Curve of Serum and Salivary CA15-3.

A: Correlation Curve of Serum CA15-3 and Salivary CA15-3 by ELISA in breast cancer patients (r=0.56; p=0.0047). (r=correlation coefficient). B: Correlation Curve of Serum CA15-3 and Salivary CA15-3 by CLIA in breast cancer patients (r= 0.36, p=0.19). C- Correlation Curve of Serum CA15-3 and Salivary CA15-3 by ELISA in healthy controls (r=0.18 p=0.35), D- Correlation Curve of Serum CA15-3 and Salivay CA15-3 by CLIA in healthy controls (r=0.08 p=0.77). ELISA: Enzyme-linked immunosorbent assay, CLIA: chemiluminescence assay

4 DISCUSSION

We analyzed serum and saliva samples of 28 healthy subjects and 26 breast cancer patients. Serum CA15-3 was detected by ECLIA and salivary CA15-3 was detected by ECLIA, CLIA, and ELISA. Previous studies have reported detection of CA15-3 with ELISA but not with ECLIA and CLIA (19, 20, 22-24). We chose to evaluate detection of CA15-3 using CLIA and ECLIA because these techniques are used routinely in clinical exams for

evaluation of serum tumor markers and serology of viral infectious agents (28). Recently, CLIA was used to evaluate proteins in liquor, demonstrating that the method can be used to analyze different fluids, such as saliva (29). ECLIA and CLIA do not require long incubations or the addition of stopping reagents, so they have superior low-end sensitivity, and a faster protocol than conventional colorimetric assays such as ELISA.

Several hypothetical mechanisms have been raised to explain the presence of large molecules such as CA15-3 in saliva. The proposed hypothesis is that active transport of proteins into saliva by the salivary glandular epithelium could explain the presence of membrane-bound proteins such as CA15-3. In the presence of breast cancer, there would be an overabundance of various bioactive proteins associated with the rapid, abnormal growth of the neoplasm, which in turn could produce a response in the salivary glands (30). However, further studies are necessary in order to better understand the regulatory mechanisms of elevated salivary CA15-3 in breast cancer patients.

Luminal subtype breast cancer shows a higher expression of MUC1 genes and a positive relationship between MUC1 and estrogen receptor (ER) gene expression has been reported (31). Park *et al*. reported higher values for CA15-3 in luminal subtypes of tumor than in other subtypes (32). Our results showed the highest values for serum and salivary CA15-3 for luminal subtypes of breast cancer.

In many tumor types, MUC1 expression correlates with aggressive, metastatic disease, poor response to therapy, and poor survival (33). MUC1 expression is seen in all subtypes of breast cancer, including luminal, HER2, and basal, although in each of these cancer types, expression is highest in tumors that have metastasized (34). The detection of CA15-3 in patient sera is currently used as a marker of response to therapy and as a prognostic indicator for survival (35). In fact, the serum antigen CA15-3 is one of the most widely used serum antigens in cancer, with high CA15-3 levels correlating with higher grade tumors, lymph node involvement, and presence of distant metastases in breast cancer (33). Emens *et al*. showed that the

concentration of serum CA 15-3 increases with increasing TNM stage, with 9% of stage I, 19% of stage II, 38% of stage III, and 75% of stage IV cases showing abnormal serum CA15-3 concentrations (36). In our samples, stage IV disease was related to the greatest mean values of CA15-3 in serum and saliva when compared with the earlier stages of disease (I–III).

In the present study, a moderate association was found between serum and salivary CA15-3 in breast cancer patients using ELISA (r=0.56; p=0.0047). Agha-Hosseini *et al*. found that salivary and serum levels of CA15-3 were significantly higher in cancer patients, with a significant positive correlation between serum and saliva CA15-3 concentrations (37). Streckfus *et al*. also reported a moderate correlation between salivary and serum CA15-3 concentration with ELISA (20).

Currently, the main clinical applications of CA15-3 in breast cancer are the preclinical detection of recurrent disease and monitoring treatment of patients with advanced disease (12). However, serum CA15-3 is an invasive exam requiring venipuncture in patients who usually have fragile veins due to previous chemotherapy and excessive routine blood tests. Salivary methods for protein detection would allow evaluation without pain and discomfort to the patient and could therefore provide a more convenient alternative to CA15-3 serum assays (38). The possibility of biomarkers using cancer derived saliva exosomes is attractive because of the stability of vesicles in blood and fluids (39).

Overall, our results confirm that serum CA15-3 values are higher in breast cancer patients, but this was not the case for salivary CA15-3. ECLIA was not a good method to detect salivary CA15-3, although it is the golden standard for detecting serum CA15-3. In breast cancer patients, we observed a correlation between serum and salivary CA15-3 detected by ELISA. CA15-3 concentrations were highest in stage IV and luminal breast cancer subtypes. Further investigations are needed to confirm the capability of detection of salivary CA15-3 and its correlation to serum CA15-3.

ACKNOWLEDGEMENTS

This work was supported by grants from *Fundação de Apoio à Pesquisa do Distrito Federal* (FAPDF, Brasília, Brazil)

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018(68):394-424.

2. Senkus E, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rutgers E, et al. Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2015;26 (Suppl 5):v8-30.

3. Steven A. Narod JI, Anthony B. Miller. Why have breast cancer mortality rates declined? Journal of Cancer Policy. 2015;5:8-17.

4. Arellano M, Jiang J, Zhou X, Zhang L, Ye H, Wong DT, et al. Current advances in identification of cancer biomarkers in saliva. Frontiers in bioscience (Scholar edition). 2009;1:296-303.

5. Giuliano AE, Connolly JL, Edge SB, Mittendorf EA, Rugo HS, Solin LJ, et al. Breast Cancer-Major changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA Cancer J Clin. 2017;67(4):290-303.

6. Berg WA, Gutierrez L, NessAiver MS, Carter WB, Bhargavan M, Lewis RS, et al. Diagnostic accuracy of mammography, clinical examination, US, and MR imaging in preoperative assessment of breast cancer. Radiology. 2004;233(3):830-49.

7. Zhang YJ, Wei L, Li J, Zheng YQ, Li XR. Status quo and development trend of breast biopsy technology. Gland surgery. 2013;2(1):15-24.

8. Duraisamy S, Kufe T, Ramasamy S, Kufe D. Evolution of the human MUC1 oncoprotein. Int J Oncol. 2007;31(3):671-7.

9. Taylor-Papadimitriou J, Burchell J, Miles DW, Dalziel M. MUC1 and cancer. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 1999;1455(2):301-13.

10. Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control of the cell surface. Nature reviews Cancer. 2004;4(1):45-60.

11. Nath S, Mukherjee P. MUC1: a multifaceted oncoprotein with a key role in cancer progression. Trends in molecular medicine. 2014;20(6):332-42.

12. Duffy MJ, Shering S, Sherry F, McDermott E, O'Higgins N. CA 15-3: a prognostic marker in breast cancer. The International journal of biological markers. 2000;15(4):330-3.

13. Harris LN, Ismaila N, McShane LM, Andre F, Collyar DE, Gonzalez-Angulo AM, et al. Use of Biomarkers to Guide Decisions on Adjuvant Systemic Therapy for Women With Early-Stage Invasive Breast Cancer: American Society of Clinical Oncology Clinical Practice Guideline. Journal of Clinical Oncology. 2016;34(10):1134-50.

14. Loo JA, Yan W, Ramachandran P, Wong DT. Comparative human salivary and plasma proteomes. Journal of dental research. 2010;89(10):1016-23.

15. Punyadeera C, Slowey PD. Chapter 22 - Saliva as an Emerging Biofluid for Clinical Diagnosis and Applications of MEMS/NEMS in Salivary Diagnostics. In: Subramani K, Ahmed W, Hartsfield JK, editors. Nanobiomaterials in Clinical Dentistry: William Andrew Publishing; 2013. p. 453-73.

16. Schafer CA, Schafer JJ, Yakob M, Lima P, Camargo P, Wong DT. Saliva diagnostics: utilizing oral fluids to determine health status. Monographs in oral science. 2014;24:88-98.

17. Gion M, Mione R, Leon AE, Dittadi R. Comparison of the Diagnostic Accuracy of CA27.29 and CA15.3 in Primary Breast Cancer. Clinical Chemistry. 1999;45(5):630.

18. Lawicki S, Bedkowska GE, Szmitkowski M. VEGF, M-CSF and CA 15-3 as a new tumor marker panel in breast malignancies: a multivariate analysis with ROC curve. Growth Factors. 2013;31(3):98-105.

19. Bigler LR, Streckfus CF, Copeland L, Burns R, Dai X, Kuhn M, et al. The potential use of saliva to detect recurrence of disease in women with breast carcinoma. J Oral Pathol Med. 2002;31(7):421-31.

20. Streckfus C, Bigler L, Dellinger T, Pfeifer M, Rose A, Thigpen JT. CA 15-3 and c-erbB-2 presence in the saliva of women. Clinical oral investigations. 1999;3(3):138-43.

21. Li P, Ye H, Liu J, Jin H, Lin Y, Yan S, et al. Evaluation of a newly developed quantitative determination kit for tumor marker CA15-3 with chemiluminescent assay. LID - 10.1002/jcla.22158 [doi]. (1098-2825 (Electronic)).

22. Streckfus C, Bigler L, Dellinger T, Dai X, Kingman A, Thigpen JT. The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: a preliminary study. Clinical cancer research : an official journal of the American Association for Cancer Research. 2000;6(6):2363-70.

23. Laidi F, Bouziane A, Lakhdar A, Khabouze S, Amrani M, Rhrab B, et al. Significant correlation between salivary and serum Ca 15-3 in healthy women and breast cancer patients. Asian Pacific journal of cancer prevention : APJCP. 2014;15(11):4659-62.

24. Streckfus C, Bigler L. The use of soluble, salivary c-erbB-2 for the detection and post-operative follow-up of breast cancer in women: the results

of a five-year translational research study. Advances in dental research. 2005;18(1):17-24.

25. Monobind Inc. CA 15-3 Test System Product Code 5625-300 [cited 2019 29/06/2019]. Available from: https://system.na3.netsuite.com/core/media/media.nl?id=4038&c=445858&h=1ddb9311185c665856ca&_xt=.pdf.

26. AJCC. Cancer Staging Manual 2017. Available from: https://cancerstaging.org/Pages/default.aspx.

27. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thurlimann B, et al. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Annals of Oncology. 2013;24(9):2206-23.

28. Victer TN, Dos Santos CS, Bao SN, Sampaio TL. Deceased tissue donor serology and molecular testing for HIV, hepatitis B and hepatitis C viruses: a lack of cadaveric validated tests. Cell and tissue banking. 2016;17(4):543-53.

29. Liu B, Chen P, Song C, Li N, Tian Y, Dong Y, et al. [Establishment of a chemiluminescent immunoassay(CLIA) and its application]. Xi bao yu fen zi mian yi xue za zhi = Chinese journal of cellular and molecular immunology. 2018;34(4):315-9.

30. Streckfus CF, Bigler L, Edwards C, Guajardo-Streckfus C, Bigler SA. Using Saliva Secretions to Model Disease Progression. In: Streckfus CF, editor. Advances in Salivary Diagnostics. Berlin, Heidelberg: Springer Berlin Heidelberg; 2015. p. 187-98.

31. Atoum M, Nimer N, Abdeldayem S, Nasr H. Relationships among serum CA15-3 tumor marker, TNM staging, and estrogen and progesterone receptor expression in benign and malignant breast lesions. Asian Pacific journal of cancer prevention : APJCP. 2012;13(3):857-60.

32. Park S, Ahn HK, Park LC, Hwang DW, Ji JH, Maeng CH, et al. Implications of different CA 15-3 levels according to breast cancer subtype at initial diagnosis of recurrent or metastatic breast cancer. Oncology. 2012;82(3):180-7.

33. Horm TM, Schroeder JA. MUC1 and metastatic cancer: expression, function and therapeutic targeting. Cell Adh Migr. 2013;7(2):187-98.

34. Mukhopadhyay P, Chakraborty S, Ponnusamy MP, Lakshmanan I, Jain M, Batra SK. Mucins in the pathogenesis of breast cancer: implications in diagnosis, prognosis and therapy. Biochimica et biophysica acta. 2011;1815(2):224-40.

35. Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. Journal of Clinical Oncology. 2007;25(33):5287-312.

36. Emens LA, Davidson NE. The follow-up of breast cancer. Seminars in oncology. 2003;30(3):338-48.

37. Agha-Hosseini F, Mirzaii-Dizgah I Fau - Rahimi A, Rahimi A. Correlation of serum and salivary CA15-3 levels in patients with breast cancer. (1698-6946 (Electronic)).

38. Kaczor-Urbanowicz KE, Martin Carreras-Presas C, Aro K, Tu M, Garcia-Godoy F, Wong DT. Saliva diagnostics - Current views and directions. Exp Biol Med (Maywood). 2017;242(5):459-72.

39. Dawes C, Wong DTW. Role of Saliva and Salivary Diagnostics in the Advancement of Oral Health. J Dent Res. 2019;98(2):133-

SUPPORTING INFORMATION

Supplementary Table 1. Subjects Charactheristics.

Healthy controls	Age	Stage	Body Mass Index	Systemic disease	Use of medication	Menopause	Tobacco use	Molecular subtype	Serum CA15.3 U/mL	Salivary CA15.3 CLIA U/mL	Salivary CA 15.3 ELISA U/mL
HC 1	28	N/A	20.45	no	Birth control pills, pantogar, pantoprazole	no	no	N/A	**20.9**	**1.51**	**1.35**
HC 2	24	N/A	21.97	no	Birth control pills	no	no	N/A	**18.6**	**4.29**	1.43
HC 3	21	N/A	24.98	no	no	no	no	N/A	**17.9**	**4.69**	1.77
HC 4	35	N/A	20.06	no	no	no	no	N/A	**22.5**	**<1.0**	1.16
HC 5	29	N/A	20.32	no	no	no	no	N/A	**14.0**	**4**	2.30
HC 6	26	N/A	23.63	no	Birth control pills	no	no	N/A	**8.6**	**<1.0**	0.76
HC 7	26	N/A	18.55	no	Birth control pills	no	no	N/A	**7.5**	**<1.0**	0.84
HC 8	22	N/A	20.96	no	no	no	no	N/A	**4.7**	**1.42**	1.32
HC 9	25	N/A	19.42	no	no	no	no	N/A	**14.7**	**<1.0**	1.16
HC 10	44	N/A	25.89	no	no	no	no	N/A	**23.8**	**<1.0**	1.35

HC 11	31	N/A	22.76	no	no	no	no	N/A	**16.5**	**<1.0**	1.38
HC 12	50	N/A	22.1	no	omeprazole, zirvit, flebon	yes	no	N/A	**11.5**	**2.43**	1.19
HC 13	31	N/A	19.38	hipothyroidism	Levothyroxine	no	no	N/A	**14.2**	**<1.0**	0.97
HC 14	47	N/A	24.24	hipothyroidism	Levothyroxine	no	no	N/A	**16.2**	**<1.0**	1.22
HC 15	22	N/A	24.98	no	Birth control pills	no	no	N/A	**20.3**	**<1.0**	0.95
HC 16	39	N/A	28.55	no	no	no	no	N/A	**4.5**	**2.05**	1.43
HC 17	31	N/A	22.04	no	no	no	no	N/A	**23.4**	**5.52**	2.02
HC 18	46	N/A	22.4	no	no	no	no	N/A	**14.5**	**<1.0**	1.41
HC 19	39	N/A	25.2	diabetes	insuline	no	no	N/A	**14.9**	**15.3**	3.47
HC 20	37	N/A	22.1	no	no	no	no	N/A	**14.7**	**8.05**	1.35
HC 21	38	N/A	28.7	diabetes	glifage	no	no	N/A	**19.5**	**1.32**	1.24
HC 22	73	N/A	17.58	depression	mirtazapine, trazadone	yes	no	N/A	**27.0**	**2.83**	1.51
HC 23	55	N/A	23.23	no	no	yes	no	N/A	**22.8**	**3.31**	1.32
HC 24	39	N/A	25.3	no	no	no	no	N/A	**7.3**	**<1.0**	1.35
HC 25	37	N/A	30.5	dislipidemia	sinvastatin	no	no	N/A	**7.5**	**13.4**	2.36
HC 26	74	N/A	20.8	hypertension, dislipidemia, hipothyroidism	Losartan, Levothyroxine, sinvastatin	yes	no	N/A	**21.6**	**27.5**	12.14

HC 27	44	N/A	25.09	hypertension	Birth control pills	no	no	N/A	**19.9**	**<1.0**	1.14
HC 28	41	N/A	20.88	no	no	no	no	N/A	**10.8**	**<1.0**	1.41

Breast Cancer patients	**Age**	**Stage**	**Body Mass Index**	**Systemic disease**	**Use of medication**	**Menopause**	**Tobacco use**	**Molecular subtype**	**Serum CA15.3 U/mL**	**Salivary CA15.3 CLIA U/mL**	**Salivary CA 15.3 ELISA U/mL**
BC 1	34	IIA	28.63	no	no	no	yes	luminal A	39.5	1.1	1.14
BC 2	42	IV	23.53	no	no	yes	no	luminal B HER2+	351.1	23.8	5.14
BC 3	51	IIA	26.69	hypertension, diabetes	Losartan, metformin, Indapamide	yes	no	TN	15.3	9.5	0.95
BC 4	77	IV	30.86	hiperthyroidism	Duspatalin, Sertraline, clonazepam, atorvastatin, Tapazole	yes	no	Luminal B HER2-	65.0	2.1	1.24
BC 5	30	IIA	22.77	no	no	no	no	TN	11.1	1.8	1.46
BC 6	39	IIA	20.7	no	no	no	no	luminal A	20.2	1.1	1.38
BC 7	42	I	21.91	no	no	no	yes	luminal A	8,5	<1.0	1.27
BC 8	48	IIA	31.25	hypertension, hipothyroidism	losartan, Atenolol, levothyroxine	no	yes	luminal A	17.0	<1.0	1.35
BC 9	56	IIA	26.57	hypertension,	valsartan, levothyroxine	yes	no	HER2 +	12.9	2.7	1.27

				hipothyroidism							
BC 10	68	I	21.61	hipothyroidism	levothyroxine	yes	no	TN	24.5	<1.0	1.54
BC 11	42	IIIB	22.41	no	Hormone replacement therapy	yes	no	?	210.0	1.1	1.35
BC 12	37	IIB	33.2	no		no	no	luminal A	16.1	<1.0	1.35
BC 13	56	IIIB	20.89	no	no	yes	yes	luminal A	19.0	<1.0	1.24
BC 14	54		18.73	no		yes	no	?	16.2	<1.0	1.32
BC 15	35	IIA	19.11	no	no	no	no	luminal B HER2-	N/A	6.9	1.87
BC 16	53	IIIB	28.98	hypertension	Amlodipine, Hydroclorothiazide	yes	no	luminal B HER2+	N/A	1.4	1.11
BC 17	47	IIB	23.73	no	no	no	no	HER2 +	9.1	4.6	1.71
BC 18	71	IV	29.07	hypertension	Captopril	yes	no	Luminal B HER2-	328.8	1.3	1.71
BC 19	55	IIA	23.2	hypertension	atenolol	no	no	HER2 +	18,3	2.4	4.22
BC 20	51	IV	24.2	no	no	yes	no	luminal A	1766.0	10.2	4.22
BC 21	37	IIA	25.5	hypertension	Atenolol	no	no	TN	9.0	<1.0	1.41
BC 22	51	IIIB	20.8	no	no	no	no	luminal B HER2+	62.4	<1.0	1.41

BC 23	53	IIIC	32.9	no	no	yes	no	TN	N/A	<1.0	1.05
BC 24	39	IIA	28	no	no	no	no	luminal B HER2+	11.3	6.5	2.64
BC 25	42	IV	25.1	no	no	no	no	HER2 +	30.1	1.5	1.32

BC: breast cancer patient, TN: triple negative, HC: healthy control

Supplementary Table 2. Serum (ECLIA) and salivary (CLIA and ELISA) values of CA15-3 for each subject.

Breast Cancer patients	Serum CA15.3 U/mL	Salivary CA15.3 CLIA U/mL	Salivay CA 15.3 ELISA U/mL	Healthy controls	Serum CA15.3 U/mL	Salivay CA15.3 CLIA U/mL	Salivay CA 15.3 ELISA U/mL
BC1	39.5	1.1	1.14	HC 1	20.9	1.51	1.35
BC 2	351.1	23.8	5.14	HC 2	18.6	4.29	1.43
BC 3	15.3	9.5	0.95	HC 3	17.9	4.69	1.77
BC 4	65.0	2.1	1.24	HC 4	22.5	<1.0	1.16
BC 5	11.1	1.8	1.46	HC 5	14.0	4	2.30
BC 6	20.2	1.1	1.38	HC 6	8.6	<1.0	0.76
BC 7	8,5	<1.0	1.27	HC 7	7.5	<1.0	0.84
BC 8	17.0	<1.0	1.35	HC 8	4.7	1.42	1.32
BC 9	12.9	2.7	1.27	HC 9	14.7	<1.0	1.16
BC 10	24.5	<1.0	1.54	HC 10	23.8	<1.0	1.35
BC 11	210.0	1.1	1.35	HC 11	16.5	<1.0	1.38
BC 12	16.1	<1.0	1.35	HC 12	11.5	2.43	1.19
BC 13	19.0	<1.0	1.24	HC 13	14.2	<1.0	0.97
BC 14	16.2	<1.0	1.32	HC 14	16.2	<1.0	1.22
BC 15	N/A	6.9	1.87	HC 15	20.3	<1.0	0.95
BC 16	N/A	1.4	1.11	HC 16	4.5	2.05	1.43
BC 17	9.1	4.6	1.71	HC 17	23.4	5.52	2.02
BC 18	328.8	1.3	1.71	HC 18	14.5	<1.0	1.41
BC 19	18.3	2.4	4.22	HC 19	14.9	15.3	3.47
BC 20	1766.0	10.2	4.22	HC 20	14.7	8.05	1.35

BC 21	9.0	<1.0	1.41	HC 21	19.5	1.32	1.24
BC 22	62.4	<1.0	1.41	HC 22	27.0	2.83	1.51
BC 23	N/A	<1.0	1.05	HC 23	22.8	3.31	1.32
BC 24	11.3	6.5	2.64	HC 24	7.3	<1.0	1.35
BC 25	30.1	1.5	1.32	HC 25	7.5	13.4	2.36
BC 26	19.8	2.4	1.32	HC 26	21.6	27.5	12.14
-	----	----	----	HC 27	19.9	<1.0	1.14
-	----	----	----	HC 28	10.8	<1.0	1.41

BC: breast cancer, HC: healthy control, ECLIA: electrochemiluminescence assay, CLIA: chemiluminescence assay, ELISA: Enzyme-linked immunosorbent assay

2.2 ARTICLE 2: Salivary metabolites in cancer patients: a systematic review of diagnostic value

Daniele Xavier Assad [1, 2]

Elisa Cançado Porto Mascarenhas [1,3]

Isabela Porto[1]

Helene Chardin [4,5]

Audrey Combes[4]

Ana Carolina Acevedo[1]

Eliete Neves Silva Guerra *[1]

[1]Laboratory of Oral Histopathology, Health Sciences Faculty, University of Brasília

Campus Universitário Darcy Ribeiro, 70910-900 Brasília DF, Brazil

[2]Medical Oncology Department, Hospital Sírio-Libanês, SGAS 613 Conj. E Bl. B, Brasília DF, 70200730, Brazil

[3]Medical Oncology Department, Cettro - Centro de Câncer de Brasília, SMH/N Quadra 02, 12º Andar, Brasilia, DF, 70710-904, Brazil.

[4]Department of Analytical, Bioanalytical Sciences and Miniaturization (LSABM), ESPCI Paris, UMR CBI 8231, PSL Research University, Paris, France

[5]Faculté de Chirurgie Dentaire, Université Paris Descartes Sorbonne 12 Rue de l'École de Médecine, 75006, Paris Cité, Paris, France

ABSTRACT

This systematic review aimed to evaluate salivary metabolites and their diagnostic value in cancer patients. Five electronic databases were searched. The risk of bias in individual studies was evaluated using the revised Quality Assessment of Diagnostic Accuracy Studies criteria. Among 1,151 identified studies, 25 were included: 13 and 12 studies used targeted and untargeted metabolomics approaches, respectively. Most studies included breast and oral cancer patients. Overall, 140 significant salivary metabolites were described among patients and healthy controls. The most frequently reported metabolites were alanine, valine and leucine. Combined salivary proline, threonine, and histidine showed better discriminatory performance for breast cancer, with excellent diagnostic test accuracy (DTA) and sensitivity and good specificity. Monoacylglycerol demonstrated the highest DTA for breast cancer (0:0/14:0/0:0). Combined choline, betaine, pipecolinic acid, and L-carnitine demonstrated excellent sensitivity and specificity for early oral cancer. Research on metabolites in saliva may determine biomarkers for cancer diagnosis.

Keywords: cancer, saliva, metabolites, mass-spectrometry, amino acids, lipids.

1 INTRODUCTION

In 2018, there were an estimated 18,078,957 new cases of cancer and 9,555,027 related deaths worldwide (1). Since delays in diagnosis may increase the mortality due to disease, every effort should be made for an early diagnosis. Biopsy with histopathological examination is the standard procedure for confirming the diagnosis. Despite its efficacy, novel adjunctive screening aids are needed to reduce the morbidity and mortality related to cancer.

Altered cell metabolism is the hallmark of cancer (2). Cancer cells use similar metabolic networks to that of the normal tissues from which they originate; the metabolism is altered to facilitate proliferation and survival in adverse environments (3, 4). Depending on the objectives of the study, metabolomics strategies may utilize two primary analysis approaches, namely, “untargeted-discovery-global” and “targeted-validation-tandem.” These approaches should be performed consecutively to achieve accurate identification and quantitation of the metabolites (5). Untargeted discovery metabolomics is a hypothesis-generating technique and allows a full scanning of the metabolome for discovery. Global metabolomics profiling, metabolomics fingerprinting, or footprinting allows classification of phenotypes, determination of pathways, qualitative identification, and relative quantification (6).

A cancer biomarker refers to a substance or process that is indicative of the presence of cancer in the body; it may be a molecule released by the malignancy itself, or a specific response of the body to the presence of cancer (7). Endogenous metabolites, including nucleic acids, lipids, amino acids (AA), peptides, vitamins, organic acids, thiols, and carbohydrates, represent a valuable tool for the identification of biomarkers for various diseases, and for monitoring disease progression (8). Human saliva provides insight into the state of health. Most biomolecules present in blood or urine, including deoxyribonucleic acids (DNA), ribonucleic acids (RNA), metabolites, and microbiota may also be found in salivary secretions (9).

Previous reports have suggested that the levels of certain metabolites, namely sialic acid (SA), taurine, proline, and valine are increased in the saliva of patients with breast cancer (BC), and may serve as biomarkers (10). In head

and neck cancer, the salivary metabolites choline, pipecolinic acid, L-phenylalanine, and S-carboxymethyl-L-cysteine have demonstrated excellent diagnostic test accuracy (DTA) (11).

Salivary metabolite profiling may represent a novel alternative or adjunct to physical, radiological, and histopathological examinations for diagnosing cancer and may provide biomarkers for diagnosing and monitoring disease progression in these patients. Many studies on saliva metabolites in cancer patients have been reported, but the diagnostic value of saliva metabolites is still uncertain. Therefore, this review aimed to evaluate the salivary metabolites and their diagnostic value in patients with cancer.

2 METHODS

This systematic review and meta-analysis adhered to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Checklist (12) .

Protocol and registration

The protocol was submitted for registration to the international prospective register of systematic reviews (PROSPERO) and is being processed under number 146372.

Study design

This was a systematic review to evaluate the role of salivary metabolites in diagnosing cancer.

Inclusion criteria

Studies that focused on the use of salivary metabolites for the diagnosis of solid malignant neoplasms were included. Studies that used saliva as the potential biological medium to diagnose and/or monitor adult patients with solid cancer using non-cancer controls, were also considered.

Exclusion criteria

Studies that evaluated volatile metabolites, did not have a healthy

control (HC) group, included tumors located in salivary glands, were not written in English, did not evaluate or identify the salivary metabolites, were not primary research articles, including reviews, letters, personal opinions, book chapters, and conference abstracts, and those that did not have available full texts, were excluded.

Information sources and search strategy

For the literature search, an individual strategy was developed for each of the following databases: Lilacs, Livivo, PubMed, Scopus, and Web of Science (Supplementary Table 1). A partial gray literature search was also performed using Google Scholar and ProQuest, and references cited in eligible articles were cross-checked. The searches were performed across all databases on January 14, 2019, with no time restriction (Supplementary Table 1). Studies were collected using reference manager software (EndNote™ Online, Thomson Reuters, Philadelphia, PA, USA). Duplicate studies were identified using the software, and any duplicates not identified by EndNote were obtained using Rayyan QCRI, a free web and mobile app for systematic reviews (Qatar Computing Research Institute, Doha, Qatar).

After identification of salivary metabolites from the selected articles, the Human Metabolome Database (HMDB) was searched manually to obtain metabolite identification and other related data; the names of the metabolites catalogued in that database were used in the search review. In cases where the metabolites lacked HMDB identification, additional searches were performed in the following databases: Lipid Maps Lipidomics Gateway, METLIN, KEGG, and PubChem; this was performed to identify any additional classes of the identified salivary metabolites and related pathways. The metabolites lacking compound identification were excluded from the analysis.

Study selection

Study selection was completed in two phases. In phase-1, two authors (D.X.A. and E.N.S.G) independently screened the titles and abstracts identified in all electronic databases, and selected articles that appeared to meet the inclusion criteria based on their abstracts. In phase-2, full articles were

evaluated to determine whether they fulfilled all and none of the inclusion and exclusion criteria, respectively. D.X.A. and E.N.S.G participated independently in phase 2. The reference lists of all included studies were critically assessed by one reviewer (D.X.A) for any inadvertently omitted references. Disagreements between the two authors were resolved by consensus. In cases where a consensus was not achieved, a third author (A.C.A.) was involved to obtain a final decision. Final selection was always based on the full text of the publication.

Data collection process

One author (D.X.A) collected the required information from the selected articles; a second author (E.N.S.G) cross-checked all data to verify the quality of data extraction. Any disagreements in either phase were resolved by discussion with a third author (A.C.A.). A fourth reviewer (H.C.) was involved as required, to enable formulation of the final decision. The following information was collected from all included studies: year of publication, author(s), country, tumor location, sample size (cases of cancers and healthy controls), saliva collection method, metabolic identification method, metabolomic strategies, metabolites identified, p-values, and main conclusions. In cases where the required data were not complete, attempts were made to contact the authors to retrieve the missing information.

Risk of bias in individual studies

The methodology of the selected studies' was evaluated independently by two authors (D.X.A. and E.N.S.G) using the critical review checklist of the revised Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) (13). These authors scored each item with a low and high bias risk as "yes" and "no" or "unclear," respectively. Disagreements were resolved by a third reviewer (A.C.A.).

Additional analyses

DTA tables were constructed using the data extracted from each article, and included all accuracy measurements, namely, sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood, negative

likelihood, diagnostic odds ratio, and Youden's Index. The cut-off values that were used to interpret these data have been presented in Supplementary Table 2.

3 RESULTS

Study Selection

In phase 1 of this review, 1,243 studies were retrieved across the 5 electronic databases; among them, 1,151 articles remained after excluding the duplicates. A total of 5 studies were included from gray literature. One additional article was identified from the reference lists of the retained studies. On comprehensive evaluation of the titles and abstracts, 40 studies were included during phase 1 of the selection. In phase-2, the selection process led to the exclusion of 15 studies (Supplementary Table 3); therefore, 25 articles were retained for final analysis. The flow chart describing the process of identification, inclusion, and exclusion of the studies has been presented in Figure 1.

Study characteristics

The selected studies were published in the past 24 years (1994-2018), and were conducted in different countries including China [18, 32-35, 37, 38], Finland (25), India (14, 17, 22, 27, 28, 31), Italy (15, 19), Japan (16, 20, 21, 26, 29, 30), Mexico (36), and the USA (23, 24).

All of the studies evaluated salivary metabolites in adults; however, one study had evaluated both, saliva and biopsy tissue (20), and one study had included additional serum samples (31). The sample sizes ranged from 10 to 117, with similar numbers of controls. The vast majority of studies included patients with breast (29, 30, 37, 38) and oral cancer (14, 15, 17, 20-23, 26-29, 31-35).

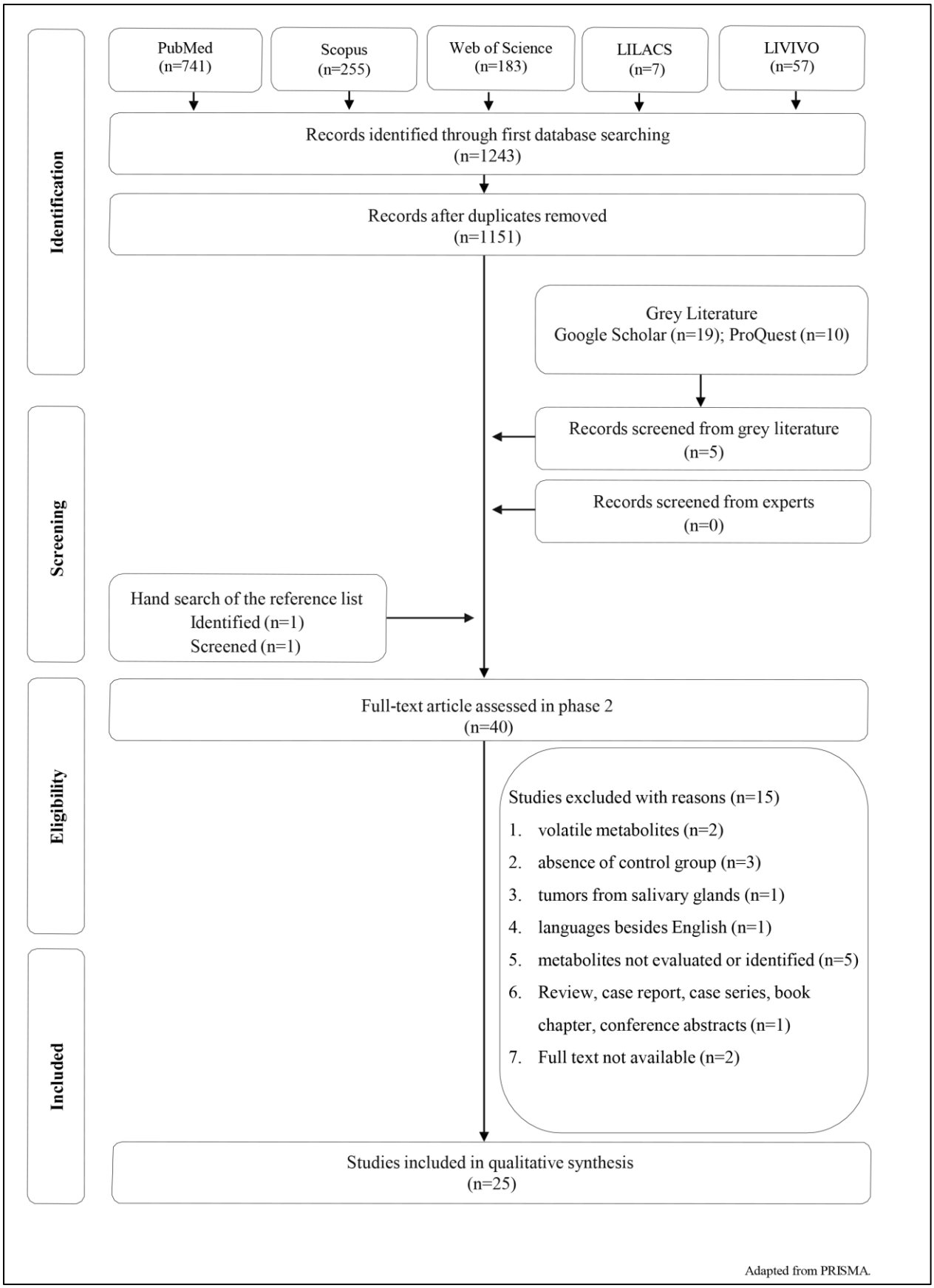


Figure 1. Flow Diagram of Literature Search and Selection Criteria.

The methods for collection of saliva included the draining method, Salivette® and spit techniques. The salivary metabolites were evaluated using Lowry's method, capillary electrophoresis mass spectrometry (CE-MS), diphenylamine method, enzyme-immunoassay (EIA), high performance liquid chromatography (HPLC), histochemical method proposed by Yao, radioimmunoassay (RIA), High Performance Liquid Chromatography-Mass Spectrometry (HPLC/MS) analysis, nuclear magnetic resonance (NMR) spectroscopy, and surface- enhanced Raman spectroscopy (87). The salivary metabolite subclasses included alcohols, amines, carbohydrates, fatty acyls, glycerophospholipids, and AA, among others. Except for the study by Zermeno-Nava et al., which was a prospective cohort study (36), all others were case control studies. A total of thirteen studies used an untargeted approach for data analysis, whereas the remainder employed a targeted approach.

In terms of the level of significance on statistical analysis, 3 studies considered differences in salivary metabolites in patients with BC and HC to be significant at $p<0.001$ (14, 28, 36); 1 study used a value of $p<0.004$ (15), while another considered $p<0.01$ to be significant (22). Two studies did not provide p-values, and the remaining 18 studies considered $p<0.05$ to be significant (16, 17, 19-21, 23-27, 29, 31-35, 37, 38]) A summary of the descriptive characteristics of the studies have been presented in Table1.

Table 1. Summary of descriptive characteristics of the included studies (n = 25)

Author/Year/ Country	**Type of Tumor**	**N of cases / N of HC**	**Metabolite Identification Method**	**Metabolomics Analysis**	**Studied Metabolites**	**Main Results**
Achalli et al., 2017, India (14)	Oral Cancer	30 (K) 30 (HC)	Diphenylamine Method	Targeted	N-Acetylneuraminate (Sialic Acid)	Mean salivary sialic acid level in subjects with OC was significantly increased when compared to the HC. Serum SA levels were elevated in OC patients
Almadori *et al*., 2007, Italy (15)	Oral Cancer, Pharyngeal cancer and Laryngeal cancer	50 (K) 77 (HC)	HPLC	Targeted	Glutathione and Uric Acid	Patients with oral or pharyngeal cancer had significantly higher salivary glutathione than both HC and patients with laryngeal cancer. No difference for uric acid in cancer groups and HC.

Asai *et al*., ,2018, Japan (16)	Pancreatic Cancer	39 (K) 26 (HC)	CE/MS	Targeted	Alanine, N1 –acetylspermidine, 2-oxobutyrate, and 2-hydroxybutyrate	Spermine, N1 - acetylspermidine, N1 - acetylspermine, and 2-aminobutanoate, showed significant differences between HC and PC.
Bahar *et al*., 2007, Israel (17)	Oral cancer	25 (K) 25 (HC)	EIA	Targeted	Uric acid	Salivary uric acid was reduced in OC patients..All salivary RNS analyzed were significantly higher and all salivary antioxidants significantly reduced in OC patients.
Chen *et al*., 2018, China (18)	Gastric Cancer	20 EGC patients, 84 AGC patients and 116 HC	HPLC-MS	Untargeted	Taurine, Glycine, Glutamine, Ethanolamine, Histidine, Alanine, Glutamic acid, Hydroxylysine, Proline, Lysine	Ten amino acids were identified as potential biomarkers, and their combination showed a promising potential for distinguishing EGC and AGC from HC.

Cheng *et al*., 2015, China (38)	Breast Cancer	27 (K) 28 (HC)	UPLC–MS	Untargeted	Arginine, Ornithine, Citrulline, Alanine, Methionine, Glutamine, Aspartic acid, Phenylalanine, Tryptophan, Proline, Threonine, Serine, Histidine, Leucine, Valine, Glutamic acid, Lysine	Concentrations of 15 SFAAs demonstrated significant differences between BC patients at stages I-II and HC. There were no significant differences in concentrations of SFAA between BCs I–II and BCs III–IV. As single salivary biomarker Pro proved the highest accuracy in predicting BC stage I-II .The diagnostic potential of 15 SAAFs as early diagnostic biomarkers for BC were verified.
Garcia *et al*., 2018, Italy (19)	Glioblastoma	10 (K) 120 (HC)	NMR Spectroscopy Analysis	Untargeted	Leucine, Valine, Isoleucine, Propionate, Alanine, Acetic acid, Ethanolamine and Sucrose	Leucine, valine, isoleucine, alanine, ethanolamine and sucrose were more concentrated in HC, whereas propionate and acetate were more concentrated in cancer

						patients.
Ishikawa *et al*., 2016, Japan (20)	Oral Cancer	24 (K) 44 (HC)	CE/TOFMS	Untargeted	Gly-Leu, N.N-Dimethylglycin, Hexanoate, Octanoate, 4-Methilbenzoate, 3PG, 3-Phenylpropionate, Isopropanolamine, SAM, 3- Phenyllactate, urea, Pipecolate, 3-(4-Hydroxyphenyl) propionate, Spermidine, butanoate, Methionine, 2-Hydroxy-4 methylpentanoate, 2-hydroxypentanoate, N-acetylornithine, 2-Aminobutyric acid , N8-acetylspermidine, Guanosine, Valine, Trimethylamine N-oxide, Trp, , 7-Methylguanine, Gly-Gly, γ-Butyrobetaine, Ala-Ala, Hypoxanthine, Ru5P, 1,3-Diaminopropane, Guanine, β-Alanine, Taurine, Choline, 3-Hydroxybutyrate, Cadaverine, O-Phosphoserine, F6P, cis-Aconitate, N-epsilon-Acetyllysine, Threonine	The concentrations of lactic acid, arginine, ornithine, adenosylmethionine and S-adenosylhomocystenine were significantly elevated, whereas glyceraldehyde 3-phosphate (3PG) and phosphoenolpyruvate (PEP) were significantly decreased in the OC group. No significantly difference in salivary metabolites in the comparison of early and advanced stages of cancer. No histological type-specific difference regarded the metabolites profile.
Ishikawa *et al*.,	Oral cancer	22 (K)	CE/MS	Untargeted	N,N-Dimethylglycine	51 metabolites differed

2017, Japan (21)	44 (HC)		Trimethylamine N-oxide, Isopropanolamine, Guanosine, Cystine, Hypotaurine, Ethanolamine Phosphate, Inosine, 5-Aminolevulinate, Gly-Leu, 3-Phenylpropionate, Cytosine, Malate, N-Acetylneuraminate (silica acid), Lysine, Ornithine, R5P, Nicotinate, Hexanoate, 3-(4-Hydroxyphenyl) Propionate, 3-Phenyllactate, 2-Hydroxy-4-methylpentanoate, Methionine, Pipecolate, , SAM, 2-Hydroxypentanoate, 2-Aminobutyric acid, Valine, Trp, N8-Acetylspermidine, N-Acetylornithine, γ-Butyrobetaine, Hypoxanthine, Ala–Ala, Gly–Gly, 7-Methylguanine, Choline,, Ru5P, Citrate, β-Ala, 3-Hydroxybutyrate, Cadaverine, Taurine, 1,3-Diaminopropane, cis-Aconitate, Carbamoylaspartate, Guanine, O-Phosphoserine, Threonine, Alanine, Leucine, Isoleucine, Glu, DHAP, F6P, Adenosine, N-ε-	significantly in controls vs. OC patients at the 12-h fasting time point. Fifteen and ten metabolites differed significantly at the 1.5- and 3.5 hours time points, respectively.. Six metabolites were consistently different from HC: N ,N –dimethylglycine, trimethylamine N –oxide, isopropanolamine, guanosine, lutamic and hypotaurine. The 12 hours fasting after dinner time point is optimal for saliva collection.

					Acetyllysine, Asp	
Jacob *et al*., 2016, India (22)	Oral Cancer	20 (K) 20 (HC)	Histochemical Method of Yao	Targeted	Total N-Acetylneuraminate (Sialic Acid)	OC patients had very high levels of salivary sialic acid levels in comparison with HC. Salivary SA levels were higher in well-differentiated squamous cell carcinoma against moderately differentiated tumors.
Lohavanichbut *et al*., 2018, USA (23)	Oral Cancer	101 (K) 35 (HC)	NMR and three types of LC-MS	Untargeted	Glutamine, Glycine, Glucose, Proline, Succinate, Isoleucine, Glutamic acid, Lactic acid, Tyrosine, Valine, Leucine, and Alanine	12 metabolites (glutamine, glycine, glucose, proline, succinate, isoleucine, glutamic acid, lactate, tyrosine, valine, leucine, and alanine) were significantly different as measured by the targeted aqueous and NMR platforms. Three metabolites (proline, glutamine, and lactic acid) were consistently different between OC and HC across all three

						platforms.
Malone *et al*., 1994, USA (24)	oral cancer, Laryngeal cancer.	51 (K) 27 (HC)	RIA	Targeted	Leucotriene B4	LTB4 levels were significantly increased in the saliva of patients with cancer compared with HC. Comparisons of PGE2 and 15-HETE levels did not reveal a significant difference in patients with cancer. Patients with advanced stage tumors (III or IV) had increased levels of LTB4 compared with patients with early stage tumors (I or II).
Mikkonen *et al*., 2018, Finland (25)	Laryngeal cancer, Oral cancer	8 (K) 30 (HC)	NMR Spectroscopy	Untargeted	Proline, 1,2-propanediol and Fucose	The median concentrations of fucose and 1,2-propanediol were significantly higher in the cancer patients compared to the HC. Instead, the proline

						was significantly lower in cancer saliva samples compared to HC. In respect of other metabolites, no statistically significant differences were observed.
Ohshima *et al*., 2017, Japan (26)	Oral Cancer	22 (K) 21 (HC)	CE-MS	Untargeted	Choline, p-hydroxyphenylacetic acid, 2-hydroxy-4 methylvaleric acid, valine, 3-phenyllactic acid, leucine, hexanoic acid, octanoic acid, terephthalic acid, [gamma]-butyrobetaine, 3-(4-hydroxyphenyl) propionic acid , isoleucine, tryptophan, 3-phenylpropionic acid, 2-hydroxyvaleric acid, butyric acid, cadaverine, 2-oxoisovaleric acid, N6,N6,N6-trimethyllysine, taurine, glycolic acid, 3-hydroxybutyric acid, heptanoic acid, alanine, and urea	A total of 25 salivary metabolites were identified as potential markers that could be used to discriminate between individuals with OC and HC. Choline showed the greatest statistically significant difference between OC patients and HC in the present study. Urea was the only metabolite that exhibited a lower level in patients with OC compared with HC. The authors used artificial neural networks to confirm the metabolites

						identifications
Reddy *et al*., 2012, India (27)	Oral Cancer	16 (K) 8 (HC)	HPLC	Untargeted	Aspartic acid, Glutamic acid, Serine, Hisitidine, Glycine, Threonine, Alanine, Arginine, Tyrosine, Valine, Methionine, Phenylalanine, Isoleucine, Leucine, Lysine	Salivary levels of amino acids were higher in both well and moderated diferentiated groups of OC patients than the HC, except for glutamic acid (0.168 µmol/mL), which was lower in well differentiated OC patients than in HC (0.222 µmol/mL).
Sanjay *et al*., 2008, India (28)	Oral Cancer	30 (K) 30 (HC)	Method of Lowry	Targeted	Total Protein, Total Sugar, Protein-Bound Sialic Acid, and Free Sialic Acid	The salivary free sialic acid levels were found to be significantly higher in well-differentiated squamous cell carcinoma than in moderately differentiated carcinoma. Protein-bound sialic acid, total proteins, and total sugars did not show any statistical

						significance between well and moderately differentiated carcinomas
Sugimoto *et al*., 2010, Japan (29)	69 Oral, 18 Pancreatic and 30 Breast Cancer	117 (K) 87 (HC)	CE-TOF-MS	Untargeted	Cadaverine, Alpha-Aminobutyric acid, Alanine, Putrescine, Methylimidazoleacetic acid, Trimethylamine, , Piperidine, Taurine, Piperideine, Pipecolic acid, Pyrroline hydroxycarboxylic acid, Betaine, Leucine + Isoleucine, Phenylalanine, Tyrosine, Histidine, Proline, Lysine, Glycine, Ornithine, Pro-Gly-Pro or Pro-Pro-Gly, , Burimamide, Ethanolamine, Gamma-Aminobutyric acid, Aspartic acid, Valine, Tryptophan, Beta-Alanine, Citrulline, Glutamic acid, Threonine, Serine, Glutamine, Hypoxanthine, Choline, Carnitine, Glycerophosphocholine,	28 metabolites discriminate between OC and HC with p <0.05. 28 metabolites for BC and 48 for PC also discriminate from HC with p<0.05. Taurine, piperidine were OC-specific markers (p<0.05) and eight metabolites (leucine-isoleucine, tryptophan, valine, glutamic acid, phenylalanine, glutamine and aspartic acid) were PC specific markers. There were no differences in metabolites between BC and other cancers.
Takayama *et*	Breast Cancer	111 (K)	UPLC-MS/MS	Targeted	Ornithine, Diaminopropane, Putrescine,	The concentrations of 10

al., 2016, Japan (30)		61 (HC)	analysis		Cadaverine , Spermidine, Spermine, N1-acetylputrescine, N1-acetyl-spermidine, N8-acetyl-spermidine, N1-acetyl-spermine, N1N8-diacetyl-spermidine, and N1N12-diacetyl-spermine	from 12 polyamines studied tended to be higher in the BC patients than those of the HC, with the exception of ornithine and putrescine that were high in HC. The profile of the ratios of the polyamines after operation of the breast tumor returned to the levels of the healthy persons
Vajaria *et al*., 2013, India (31)	Oral Cancer	100 (K) 100 (HC)	Method of Lowry	Targeted	Total protein, Total Sialic Acid	TSA/TP ratios in patients with OC were significantly higher than in HC. Salivary SA was higher in OC patients who either had chewing or/and smoking or/and snuffing or/and alcohol drinking habits
Wang *et al*., 2014, China	Oral Cancer	30 (K) 30 (HC)	UPLC–MS in Hydrophilic	Targeted	Choline, Betaine, Pipecolinic acid, L-carnitine	Four potential salivary biomarkers demonstrated

(34)			Interaction Chromatography Mode			significant differences in concentrations between patients at stages I–II and the HC.
Wang *et al*., 2014, China (33)	Oral Cancer	30 (K) 60 (HC)	UPLC–MS	Targeted	L-phenylalanine and L-leucine	L-phenylalanine and L-leucine demonstrated significant differences in concentrations between OC patients and HC. Compared to the HC, their contents were lower in the OC patients
Wang *et al*., 2014, China (32)	Oral Cancer	30 (K) 30 (HC)	UPLC-MS	Untargeted	Lactic acid, Hydroxyphenyllactic acid, N-nonanoylglycine, 5-hydroxymethyluracil, Succinic acid, Ornithine, Hexanoylcarnitine, Propionylcholine, Carnitine, 4-hydroxy-L-glutamic acid, Acetylphenylalanine, Sphinganine, Phytosphingosine, S-carboxymethyl-L-cysteine	A total of fourteen potential biomarkers have a close relationship with early stage of OC. Eight potential biomarkers were up-regulated in saliva of OC patients and six potential biomarkers were down-regulated.

Wei *et al*., 2011, China (35)	Oral Cancer	37 (K) 34 (HC)	UPLC-QTOFMS	Untargeted	c-aminobutyric acid, Phenylalanine, Valine, n-eicosanoic acid, Lactic acid, Alanine, Isoleucine, Leucine, n-Tetradecanoic acid, Proline, Phenylalanine, Threonine, n-dodecanoic acid, 3-indolepropionic acid, homocysteine, 4-methoxyphenylacetic acid	The salivary levels of GABA, phenylalanine and valine were significantly lower, while n-eicosanoic acid and lactic acid were significantly higher, in the OC group vs HC.The most significant discriminant salivary metabolites were gama-aminobutyric acid, Phenylalanine, Valine, n-eicosanoic acid and lactic acid. Valine, lactic acid and phenylalanine were also discriminated serum metabolites in OC vs HC. There was no impact of age and gender on the identified metabolites in OC patients.
Zermeno-Nava *et al*., 2018 ,Mexico	Ovarian Cancer	15 (K) 37 (HC)	SERS	Targeted	Sialic Acid	The authors concluded that if the patient presents an adnexal masses and higher salivary SA concentration

(36)						levels before surgery, there is a high probability of ovarian cancer.
Zhong *et al*., 2016, China (37)	Breast Cancer	30 (K) 25 (HC)	HPLC/MS; UPLC-MS	Untargeted	Glycerol phospholipid compounds (LysoPC (18:2), LysoPC (18:1), PS (14:1/16:1), LysoPC (16:0), LysoPC (22:6), LysoPE (18:2/0:0), PC (18:1/16:0), PE (22:0/20:4)), and the others are fatty amide (palmitic amide), sphingolipid (phytosphingosine), amino acids and its derivatives (phenylalanine, citrulline, histidine, acetylphenylalanine), choline (propionylcholine), glyceroglycolipid (MG (0:0/14:0/0:0)), saccharic acid derivative (N-Acetylneuraminic acid), and denzene pyruvic acid derivatives (4-Hydroxyphenylpyruvic acid).	Three up-regulated metabolites LysoPC (18:1), LysoPC (22:6) and MG (0:0/14:0/0:0) displayed the area under the curve values of 0.920, 0.920 and 0.929, respectively, indicating the high accuracy of this method to predict BC. Phenylalanine, citrulline and histidine were confirmed using standard samples.

N: number; K: cases of cancer; HC: controls; OC: oral cancer; HP/LC: High Performance Liquid Chromatography, CE/MS: Capillary Electrophoresis-Mass Spectrometry; PC: pancreatic cancer; NMR: Nuclear Magnetic Ressonance; EIA: Enzyme- immunoassay EIA. RNS: reactive nitrogen species; EGC: early gastric cancer; AGC: advanced gastric cancer; HPLC-MS: High Performance Liquid Chromatography-Mass Spectrometry; SFAA:

Salivary free amino acids; BC: breast cancer; UPLC-MS: Ultra-Performance Liquid Chromatography–Mass Spectrometry; CE/TOFMS: Capillary Electrophoresis Time-of-Flight Mass Spectrometry; 3PG: glyceraldehyde 3-phosphate; Trp: Tryptophan; Ru5P: ribulose 5-phosphate; F6P: fructose 6-phosphate; 5RP:ribose 5-phosphate; SAM: S-adenosylmethionine; β-Ala: β-Alanine; Glu: glutamic acid; DHAP: dihydroxyacetone phosphate; Asp: aspartic acid; LC-MS: LC-MS Liquid Chromatography Mass Spectrometry.; RIA: Radioimmunoassay; LTB4: Leukotriene B4; PGE2: prostaglandin E2; HETE: hydroxyeicosatetranoic acids; HNC: head and neck cancer; MS: mass spectrometry; SPM: spermine, CAD: cadaverine, Ac-SPM: N1-acetyl-spermine; N1-Ac-SPD: N1-acetyl-spermidine; N8-Ac-SPD: N8-acetyl-spermidine; TSA: total silica acid; TP: total protein; UPLC-QTOFMS :Ultra-Performance Liquid Chromatography–Mass Spectrometry coupled with Quadrupole/Time-of-Flight Mass Spectrometry; SERS: Surface-Enhanced Raman Spectroscopy; SA: sialic acid

Synthesis of results

The 25 included studies analyzed 140 salivary metabolites that demonstrated significant statistical differences between cancer patients and HC. Among other classes of metabolites, 46, 13, 9, 9, and 9 were AA, fatty acyls, glycerophospholipids, amines, and carbohydrates, respectively. The frequencies of the metabolite super-classes reported in the studies are shown in Figure 2A. Supplementary Table 4 shows the prevalence of the super- and sub-classes of the reported metabolites. The most frequently reported metabolites were alanine, valine, leucine, and threonine, proline, glutamic acid and phenylalanine in 11, 9, 8, and 6 studies, respectively. The most common carbohydrate in 7 studies was N-acetylneuraminate (SA), and the most common fatty acids in 3 studies were hexanoic acid and 2-hydroxypentanoate. The frequencies of the most commonly reported AA in the review are shown in figure 2B.

The metabolites were related with diverse pathways; among others, the most common were the arginine and proline, cysteine and methionine, glycine and serine, glycerophospholipid, and purine metabolic pathways. A related pathway was not found in any database or literature for 15 of 140 metabolites, while 3 were related to pathways not previously described in humans. The complete data and classification of all reported metabolites have been presented in Supplementary Table 5.

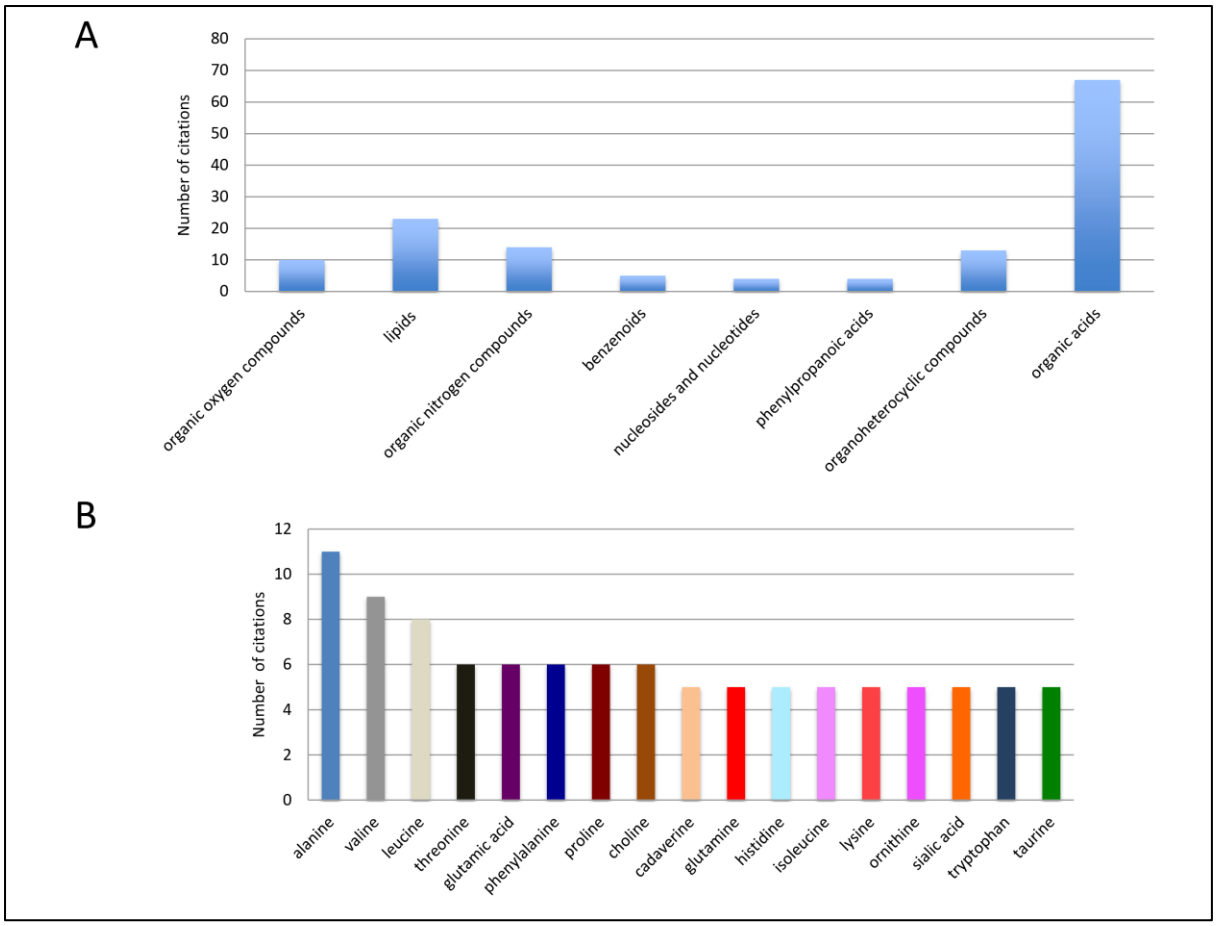


Figure 2. A. Frequency of metabolites super-classes B. Frequency of most reported amino acids in the review

Risk of bias across studies

The study methods were homogeneous; however, certain discrepancies were noted in methodological quality (case control design, lack of blinding, and unclear timing sequence of standard and index text), and none fulfilled all of the QUADAS-2 criteria. Based on the QUADAS-2 criteria, patient selection was scored at high risk of bias for all studies except that by Zermeno-Nava *et al*. (36), as the case-control design was not avoided. Additionally, for all studies, the risk of bias introduced by index test interpretation or the use of the QUADAS-2 criteria was scored as “unclear”; this was performed as information regarding blinding was lacking. Certain studies did not mention the use of the biopsy as the standard reference method for diagnosing cancer (17, 23, 26, 29, 30); the reference standard (biopsy) in these cases was not interpreted without knowledge of the results of the index text. Therefore, the reference standard and its conduct or interpretation were considered to be at high risk of introducing bias. In terms of the concerns regarding applicability, there was a

low risk of concern with respect to patient selection and reference standards and unclear concern with regard to index text. In general, based on the QUADAS-2 criteria, the selected studies were considered to have a high risk of bias, with low concerns regarding applicability . The complete list of analyzed items has been presented in Figures 3A and 3B. Supplementary Table 9 lists all the questions and responses to the QUADAS-2.

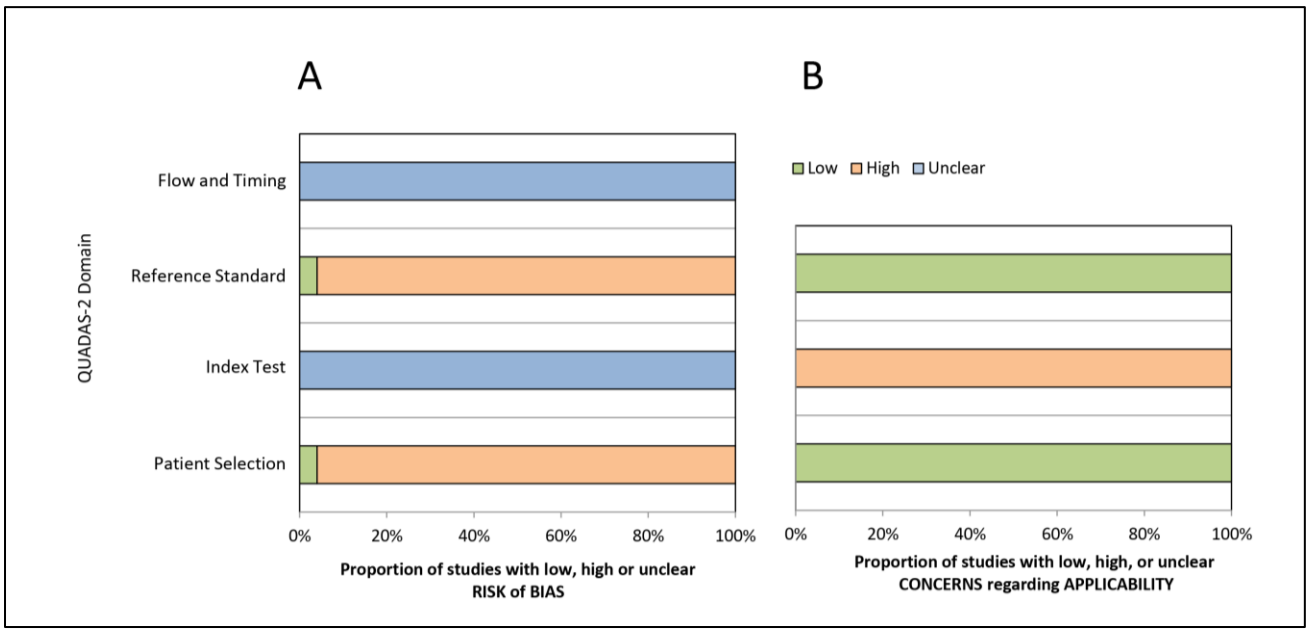


Figure 3. QUADAS-2- Quality assessment. A- Proportion of studies with low, high or unclear Risk of Bias in the domains of flow and timing, reference standard, index test and patient selection. B- Proportion of studies with low, high or unclear concerns regarding applicability in reference standard, index test and patient selection.

4 DISCUSSION

This comprehensive review on salivary metabolites and their accuracy in diagnosing cancer included 25 selected studies. Compared to HC, patients with oral, breast, laryngeal, pancreatic, ovarian, and gastric cancers and glioblastoma multiforme were found to demonstrate 140 statistically significant metabolites of different classes.

In this review, almost 50% of studies from 1994 to 2018 used a targeted approach and the remainder, conducted between 2010 and 2018, used an untargeted metabolomics approach. Only one study with an untargeted

approach confirmed all identified metabolites using a targeted approach. This is a current limitation, since untargeted metabolomics requires analysis of large volumes of data, and at present, only a fraction of the identified metabolites may be identified by their biochemical name. It is essential that this limitation is recognized, as targeted analysis should be used to validate and confirm the untargeted profile (39).

In general, NMR spectroscopy and MS (particularly LC/MS) are the two major methods used for analysis in metabolomics. The included studies that used a targeted approach were older than those that used an untargeted approach; they employed methods other than NMR spectroscopy and MS, such as the diphenylamine method (14), EIA (17), histochemical method proposed by Yao (22), RIA (24), Lowry's method [28, 31], and SERS (36). Untargeted metabolomic datasets are exceedingly complex, with file sizes of gigabytes per sample for certain new high-resolution MS instruments (6).

The process of oncogenesis is dependent on AA, the building blocks for protein synthesis, and a source of energy and metabolites (40). The essential AA may either be used for protein synthesis or be oxidized for the energy needs of tumors. In this review, 47 described metabolites were AA. All the branched-chain (essential) AA such as phenylalanine, valine, threonine, methionine, leucine, isoleucine, lysine, tryptophan and histidine were among those described in this review. The increased concentrations of various AA seen in the tumor cells may be attributed to the regulated protein synthesis in tumors; this is related to their greater need for protein synthesis owing to rapid cell proliferation. Increased activity of various AA transport systems may enhance protein synthesis in tumors. Furthermore, accessory pathways of protein synthesis may be activated to meet the demands of rapid cell proliferation in tumors.

Leucine, isoleucine, and valine are crucial nutrition signals that mediate important effects on protein synthesis, glucose homeostasis, and nutrient-sensitive signaling pathways. The role of valine has been reported in 9 studies that included breast cancer, glioblastoma, oral cancer, and pancreatic cancer; is related to the pathways of valine, leucine, and isoleucine degradation and propanoate metabolism. Valine demonstrated a sensitivity of 82% and

specificity of 75% for oral cancer. Leucine was reported to be a marker in 8 studies that evaluated breast cancer, glioblastoma, and oral cancer and has been found to be related to the pathways of valine, leucine, and isoleucine degradation. Leucine has a sensitivity of 76% and 100% and specificity of 75% and 71% for early and advanced breast cancer, respectively.

Alanine, synthesized by alanine aminotransferases using carbon from pyruvate and nitrogen from glutamate, is related to carbon metabolism. Biosynthesis of alanine has been shown to correlate with proliferation, suggesting that it may play a role in proliferative cell metabolism (41). Alanine has been described to be an important survival signal in pancreatic cancer, where stromal cells promote the proliferation and survival of pancreatic cancer cells by secreting alanine, that may be utilized in the tricarboxylic acid cycle of cancer cells (42). Alanine is related to the pathways of alanine, aspartate, and glutamate metabolism. Alanine was reported to be a marker in 11 studies that included breast cancer, gastric cancer, glioblastoma, oral cancer, and pancreatic cancer.

The tumor-promoting effects of enhanced fatty acid synthesis were first appreciated in the 1990s when fatty acid synthase expression was identified as a prognostic marker of aggressive breast cancers. In addition to synthesis, lipid breakdown also appears to be a common feature during cancer development (43). Actively proliferating tissues require fatty acids for the synthesis of structural lipids. Therefore, the induction of lipid synthesis is extremely likely to be closely related to cell growth, which is a prerequisite for cell division. In this review, the lipids MG (0:0/14:0/0:0), lysoPC (18:2), lysoPC (18:1), and lysoPC (22:6) had the highest sensitivity and specificity values for diagnosis of BC.

The elevation of phosphocholine and total choline is one of the most widely established characteristics of cancer cells. PC is both, a precursor and a breakdown product of phosphatidylcholine, the most abundant phospholipid in biological membranes (44). Choline, an organic nitrogen compound, is related to the metabolism of betaine and methionine, and to phospholipid biosynthesis. Choline was evaluated in 6 studies including breast, oral, and pancreatic

cancers; it demonstrated better DTA for advanced stages of oral cancer compared to early stages (109.33 vs. 47.25, respectively).

Among the 140 statistically significant salivary metabolites, 9 were amines. Polyamines are synthesized from arginine and s-adenosylmethionine via conversion of arginine to ornithine by arginase, and ornithine decarboxylation to putrescine, a polyamine precursor containing two amine groups, by ornithine decarboxylase (45). Polyamines have been associated with rapid tumor growth owing to their biosynthesis and accumulation in these cases, and their involvement in diverse functions related to cell growth and differentiation, such as DNA synthesis and stability, regulation of transcription, ion channel regulation, and protein phosphorylation. Cadaverine has been identified in 5 studies on oral, breast, and pancreatic cancers. Takayama *et al*. (30) found cadaverine to have a sensitivity of 67% and specificity of 73% for diagnosing breast cancer.

SA is one of the key monosaccharide building blocks that compose cell surface glycans on mammalian cells (36). These residues are strategically positioned at the tip of glycans, placing them at the forefront of many critical cellular processes involving cell–cell contact (46). Changes in glycosylation, and upregulation and alteration of terminal SA structures are classic hallmarks of malignant transformation(46). The role of SA was evaluated in 7 studies that evaluated breast, oral, and ovarian cancers. Its sensitivity and specificity for diagnosing ovarian cancer was 80% and 100%, respectively (36).

Why salivary metabolites reflect serum metabolism remains a major question. In the case of oral cancers, the plausible explanation may be that systemic biological fluids including blood and lymph circulate around these tumors; the blood containing the metabolites travels through the salivary gland, releasing the metabolites into the saliva. Oral cancers are in direct contact with the saliva; owing to local tissue destruction, glycoproteins, which are an integral part of tumor cells, are released into the saliva. These are hydrolyzed by peptidases and proteases present in the saliva, leading to an AA pool that facilitates further identification of salivary metabolites. (29). Another hypothesis suggests that systemic diseases alter salivary biomarker profiles by liberation

of tumor growth factors. Gao *et al*. used mouse models of cancer to determine whether salivary biomarker profiles are affected by distal tumor development, and suggested that the distant tumors produce growth factors, which may alter the transcriptome of the salivary glands, and consequently, the saliva. An additional hypothesis suggests the existence of systemic networks in the human body, that allow communication between distal diseases and the salivary glands. Signals transmitted through such networks may induce related signaling pathways that result in altered gene expression, and protein translation and metabolism, thereby producing disease-induced salivary biomarker profiles (47).

Intra-tumoral heterogeneity may challenge the use of a single classification model of salivary metabolites for diagnosing cancer. The heterogeneous nature of oral cancers, including oral squamous cell carcinoma, and oropharyngeal and lingual cancers may produce various metabolite profiles. The diverse molecular profiles of breast cancer may result in similar outcomes, as they comprise structurally distinct types based on the expression of hormone receptors such as estrogen and progesterone; they are also affected by clinical parameters, such as the patient's age or menopausal status (29).

In summary, compared with HC, 140 significantly different salivary metabolites were identified in the diagnosis of oral, breast, laryngeal, pancreatic, ovarian, and gastric cancer, and glioblastoma multiforme; the majority were AA. Detection of disease in the early stages is essential for the successful treatment for most cancers. Furthermore, the rapid identification of suspicious lesions may also reduce hospital burdens. In this context, salivary metabolites could facilitate a rapid, easily accessible, and non-invasive means of obtaining a clinical diagnosis; it may also be helpful during follow-up. In the long term, this will facilitate the diagnosis of a larger number of cancers in the early stages of their development or relapse, and will serve to reduce the related mortality.

Limitations

Except for the study by Zermeno-Nava (36) *et al*., all others had a case control design, that demonstrated limitations in methodological quality (case control design, lack of blinding, and unclear timing sequence of standard and index text). Only one of the studies fulfilled all QUADAS-2 methodological quality criteria. Approximately half of the included studies did not report values of DTA. Among the studies on breast cancer, the metabolites were only identified among Asian populations in 3 studies. In the study by Sugimoto *et al*., the ethnicity of the patients were not specified; therefore, these metabolites may not be generalized to all populations with BC. The size of the samples included in the studies was also a matter of concern. Among 13 studies that used a targeted approach, only 4 included at least 50 patients to confirm the hypothesis. Among the 12 studies that used an untargeted approach for hypothesis generation, only 4 included at least 50 patients. Only one study confirmed all the results of the untargeted approach; this demonstrates that metabolomics is an area with its own particularities, which include costs.

Conclusion

This review evaluated studies that utilized targeted and untargeted metabolomics approaches to identify the value of salivary metabolites in diagnosing cancer. Among 140 salivary metabolites that demonstrated statistically significant differences between cancer patients and healthy controls, 46 were AA. Proline, threonine, and histidine in combination demonstrated excellent sensitivity and specificity for diagnosing breast cancer, and choline, betaine, pipecolinic acid, and L-carnitine in combination demonstrated excellent sensitivity and specificity in the diagnosis of early oral cancers. The highest DTA for diagnosing breast cancer was for MG (0:0/14:0/0:0). Further studies are needed to compare the metabolite profiles obtained concurrently from saliva, blood, and cancer tissues; these will provide rational evidence for links among systemic metabolites. This review highlights the current evidence on salivary metabolites that may be used to diagnose cancer. Further studies including larger sample sizes with confirmation of the results of untargeted analysis, are warranted.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018(68):394-424.

2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.

3. Jang M, Kim SS, Lee J. Cancer cell metabolism: implications for therapeutic targets. Experimental &Amp; Molecular Medicine. 2013;45:e45.

4. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. Nature medicine. 2004;10(8):789-99.

5. Nalbantoglu S. Metabolomics: Basic Principles and Strategies. 2019.

6. Patti GJ, Yanes O, Siuzdak G. Innovation: Metabolomics: the apogee of the omics trilogy. Nat Rev Mol Cell Biol. 2012;13(4):263-9.

7. Wagner PD, Srivastava S. New paradigms in translational science research in cancer biomarkers. Translational research : the journal of laboratory and clinical medicine. 2012;159(4):343-53.

8. Wang X, Kaczor-Urbanowicz KE, Wong DT. Salivary biomarkers in cancer detection. Medical oncology (Northwood, London, England). 2017;34(1):7.

9. Loo JA, Yan W, Ramachandran P, Wong DT. Comparative human salivary and plasma proteomes. Journal of dental research. 2010;89(10):1016-23.

10. Porto-Mascarenhas EC, Assad DX, Chardin H, Gozal D, De Luca Canto G, Acevedo AC, et al. Salivary biomarkers in the diagnosis of breast cancer: A review. Crit Rev Oncol Hematol. 2017;110:62-73.

11. Guerra EN, Acevedo AC, Leite AF, Gozal D, Chardin H, De Luca Canto G. Diagnostic capability of salivary biomarkers in the assessment of head and neck cancer: A systematic review and meta-analysis. Oral oncology. 2015;51(9):805-18.

12. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS medicine. 2009;6(7):e1000097.

13. Whiting PF, Rutjes AW, Westwood ME, Mallett S, Deeks JJ, Reitsma JB, et al. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. Ann Intern Med. 2011;155(8):529-36.

14. Achalli S, Madi M, Babu SG, Shetty SR, Kumari S, Bhat S. Sialic acid as a biomarker of oral potentially malignant disorders and oral cancer. Indian journal of dental research : official publication of Indian Society for Dental Research. 2017;28(4):395-9.

15. Almadori G, Bussu F, Galli J, Limongelli A, Persichilli S, Zappacosta B, et al. Salivary glutathione and uric acid levels in patients with head and neck squamous cell carcinoma. Head & neck. 2007;29(7):648-54.

16. Asai Y, Itoi T, Sugimoto M, Sofuni A, Tsuchiya T, Tanaka R, et al. Elevated Polyamines in Saliva of Pancreatic Cancer. Cancers. 2018;10(2).

17. Bahar G, Feinmesser R, Shpitzer T, Popovtzer A, Nagler RM. Salivary analysis in oral cancer patients: DNA and protein oxidation, reactive nitrogen species, and antioxidant profile. Cancer. 2007;109(1):54-9.

18. Chen Y, Cheng S, Zhang A, Song J, Chang J, Wang K, et al. Salivary Analysis Based on Surface Enhanced Raman Scattering Sensors Distinguishes Early and Advanced Gastric Cancer Patients from Healthy Persons. Journal of biomedical nanotechnology. 2018;14:1773-84.

19. Garcia-Villaescusa A, Morales-Tatay JM, Monleon-Salvado D, Gonzalez-Darder JM, Bellot-Arcis C, Montiel-Company JM, et al. Using NMR in saliva to identify possible biomarkers of glioblastoma and chronic periodontitis. PloS one. 2018;13(2):e0188710.

20. Ishikawa S, Sugimoto M, Kitabatake K, Sugano A, Nakamura M, Kaneko M, et al. Identification of salivary metabolomic biomarkers for oral cancer screening. Scientific Reports. 2016;6:31520.

21. Ishikawa S, Sugimoto M, Kitabatake K, Tu M, Sugano A, Yamamori I, et al. Effect of timing of collection of salivary metabolomic biomarkers on oral cancer detection. Amino acids. 2017;49(4):761-70.

22. Jacob TV, Ramesh M, Murali S, Ramesh K, Sanjay PR, Abraham P. A non-invasive study to estimate and compare salivary sialic acid level as tumor marker in patients with pre-cancer and oral cancer. J Cancer Res Ther. 2016;12(2):634-9.

23. Lohavanichbutr P, Zhang Y, Wang P, Gu H, Nagana Gowda GA, Djukovic D, et al. Salivary metabolite profiling distinguishes patients with oral cavity squamous cell carcinoma from normal controls. PloS one. 2018;13(9):e0204249.

24. Malone JP, Snyderman CH. Arachidonic acid metabolites in saliva of patients with squamous cell carcinoma of the head and neck. Oral surgery, oral medicine, and oral pathology. 1994;77(6):636-40.

25. Mikkonen JJW, Singh SP, Akhi R, Salo T, Lappalainen R, Gonzalez-Arriagada WA, et al. Potential role of nuclear magnetic resonance spectroscopy to identify salivary metabolite alterations in patients with head and neck cancer. Oncology letters. 2018;16(5):6795-800.

26. Ohshima M, Sugahara K, Kasahara K, Katakura A. Metabolomic analysis of the saliva of Japanese patients with oral squamous cell carcinoma. Oncology reports. 2017;37(5):2727-34.

27. Reddy I, Sherlin HJ, Ramani P, Premkumar P, Natesan A, Chandrasekar T. Amino acid profile of saliva from patients with oral squamous cell carcinoma using high performance liquid chromatography. Journal of oral science. 2012;54(3):279-83.

28. Sanjay PR, Hallikeri K, Shivashankara AR. Evaluation of salivary sialic acid, total protein, and total sugar in oral cancer: a preliminary report. Indian journal of dental research : official publication of Indian Society for Dental Research. 2008;19(4):288-91.

29. Sugimoto M, Wong DT, Hirayama A, Soga T, Tomita M. Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. Metabolomics. 2010;6(1):78-95.

30. Takayama T, Tsutsui H, Shimizu I, Toyama T, Yoshimoto N, Endo Y, et al. Diagnostic approach to breast cancer patients based on target metabolomics in saliva by liquid chromatography with tandem mass spectrometry. Clin Chim Acta. 2016;452:18-26.

31. Vajaria BN, Patel KR, Begum R, Shah FD, Patel JB, Shukla SN, et al. Evaluation of serum and salivary total sialic acid and alpha-l-fucosidase in patients with oral precancerous conditions and oral cancer. Oral Surg Oral Med Oral Pathol Oral Radiol. 2013;115(6):764-71.

32. Wang Q, Gao P, Wang X, Duan Y. The early diagnosis and monitoring of squamous cell carcinoma via saliva metabolomics. Scientific Reports. 2014;4:6802.

33. Wang Q, Gao P, Cheng F, Wang X, Duan Y. Measurement of salivary metabolite biomarkers for early monitoring of oral cancer with ultra performance liquid chromatography–mass spectrometry. Talanta. 2014;119:299-305.

34. Wang Q, Gao P, Wang X, Duan Y. Investigation and identification of potential biomarkers in human saliva for the early diagnosis of oral squamous cell carcinoma. Clin Chim Acta. 2014;427:79-85.

35. Wei J, Xie G, Zhou Z, Shi P, Qiu Y, Zheng X, et al. Salivary metabolite signatures of oral cancer and leukoplakia. International journal of cancer Journal international du cancer. 2011;129(9):2207-17.

36. Zermeno-Nava JJ, Martinez-Martinez MU, Ramirez-de-Avila AL, Hernandez-Arteaga AC, Garcia-Valdivieso MG, Hernandez-Cedillo A, et al. Determination of sialic acid in saliva by means of surface-enhanced Raman spectroscopy as a marker in adnexal mass patients: ovarian cancer vs benign cases. J Ovarian Res. 2018;11(1):61.

37. Zhong L, Cheng F, Lu X, Duan Y, Wang X. Untargeted saliva metabonomics study of breast cancer based on ultra performance liquid chromatography coupled to mass spectrometry with HILIC and RPLC separations. Talanta. 2016;158:351-60.

38. Cheng F, Wang Z, Huang Y, Duan Y, Wang X. Investigation of salivary free amino acid profile for early diagnosis of breast cancer with ultra performance liquid chromatography-mass spectrometry. Clin Chim Acta. 2015;447:23-31.

39. Alonso A, Marsal S, Julià A. Analytical Methods in Untargeted Metabolomics: State of the Art in 2015. Frontiers in Bioengineering and Biotechnology. 2015;3(23).

40. Ananieva EA, Wilkinson AC. Branched-chain amino acid metabolism in cancer. Current Opinion in Clinical Nutrition & Metabolic Care. 2018;21(1):64-70.

41. Coloff JL, Murphy JP, Braun CR, Harris IS, Shelton LM, Kami K, et al. Differential Glutamate Metabolism in Proliferating and Quiescent Mammary Epithelial Cells. Cell metabolism. 2016;23(5):867-80.

42. Sousa CM, Biancur DE, Wang X, Halbrook CJ, Sherman MH, Zhang L, et al. Pancreatic stellate cells support tumour metabolism through autophagic alanine secretion. Nature. 2016;536(7617):479-83.

43. Kuhajda FP, Jenner K, Wood FD, Hennigar RA, Jacobs LB, Dick JD, et al. Fatty acid synthesis: a potential selective target for antineoplastic therapy. Proceedings of the National Academy of Sciences. 1994;91(14):6379.

44. Ackerstaff E, Glunde K, Bhujwalla ZM. Choline phospholipid metabolism: A target in cancer cells? Journal of Cellular Biochemistry. 2003;90(3):525-33.

45. Soda K. The mechanisms by which polyamines accelerate tumor spread. Journal of experimental & clinical cancer research : CR. 2011;30(1):95-.

46. Pearce OM, Laubli H. Sialic acids in cancer biology and immunity. Glycobiology. 2016;26(2):111-28.

47. Zhang Y, Sun J, Lin CC, Abemayor E, Wang MB, Wong DTW. The emerging landscape of salivary diagnostics. Periodontology 2000. 2016;70(1):38-52.

SUPPORTING INFORMATION

Supplementary Table 1. Search strategy and date that was performed in the chosen Databases.

Database	Search (January 14, 2019)
PubMed	#4 #1 AND #2 AND #3 #3 "metabolomics" OR "metabolomics biomarkers" OR metabolite OR "Metabolomics"[Mesh] OR "Tumor Metabolite Markers" OR "Tumor Metabolite Marker" OR "Neoplasm Metabolite Markers" OR "Neoplasm Metabolite Marker" #2 "saliva"[MeSH Terms] OR saliva OR salivas #1 "neoplasms"[MeSH Terms] OR neoplasms OR cancer OR Neoplasia OR Neoplasias OR Neoplasm OR Tumors OR Tumor OR Cancers OR "Malignant Neoplasms" OR "Malignant Neoplasm"
Scopus	TITLE-ABS-KEY(neoplasms OR cancer OR Neoplasia OR Neoplasias OR Neoplasm OR Tumors OR Tumor OR Cancers OR "Malignant Neoplasms" OR "Malignant Neoplasm") AND TITLE-ABS-KEY(saliva OR salivas) AND TITLE-ABS-KEY("metabolomics" OR "metabolomics biomarkers" OR metabolite OR "Tumor Metabolite Markers" OR "Tumor Metabolite Marker" OR "Neoplasm Metabolite Markers" OR "Neoplasm Metabolite Marker")
Web of Science	#4 #1 AND #2 AND #3 #3 TS=("metabolomics" OR "metabolomics biomarkers" OR metabolite OR "Tumor Metabolite Markers" OR "Tumor Metabolite Marker" OR "Neoplasm Metabolite Markers" OR "Neoplasm Metabolite Marker") #2 TS=(saliva OR salivas) #1 TS=(neoplasms OR cancer OR Neoplasia OR Neoplasias OR Neoplasm OR Tumors OR Tumor OR Cancers OR "Malignant Neoplasms" OR "Malignant Neoplasm")
LILACS	(tw:(Neoplasms OR Neoplasias OR Tumor OR cancer OR câncer OR cáncer)) AND (tw:(savila OR salivas)) AND (tw:(metabolomics OR Metabolômica OR Metabolómica OR "Neoplasm Metabolite Marker" OR "Tumor Metabolite Markers"))

LIVIVO	TI=((neoplasms OR cancer OR Neoplasia OR Neoplasias OR Neoplasm OR Tumors OR Tumor OR Cancers OR "Malignant Neoplasms" OR "Malignant Neoplasm")) AND TI=((saliva OR salivas)) AND TI=(("metabolomics" OR "metabolomics biomarkers" OR metabolite OR "Tumor Metabolite Markers" OR "Tumor Metabolite Marker" OR "Neoplasm Metabolite Markers" OR "Neoplasm Metabolite Marker")) *Without MEDLINE **Doc. Type: Article
Google Scholar	allintitle: saliva metabolomics
ProQuest	TI,AB(neoplasms OR cancer OR Neoplasia OR Neoplasias OR Neoplasm OR Tumors OR Tumor OR Cancers OR "Malignant Neoplasms" OR "Malignant Neoplasm") AND TI,AB(saliva OR salivas) AND TI,AB("metabolomics" OR "metabolomics biomarkers" OR metabolite OR "Tumor Metabolite Markers" OR "Tumor Metabolite Marker" OR "Neoplasm Metabolite Markers" OR "Neoplasm Metabolite Marker")

Supplementary Table 2. Test indicators extracted from De Luca Canto *et al(*1).

Test indicators	Data analysis	References
DOR	The value of a DOR ranges from 0 to infinity, with higher values indicating better discriminatory test performance. A value of 1 means that a test does not discriminate between patients with the disorder and those without it. Values lower than 1 point to improper test interpretation (more negative tests among the diseased).	Glas *et al* (2)
LR	LR+>3 and an LR-<0.3 – acceptable diagnostic test accuracy (DTA) LR+>10 and LR-<0.1 – excellent DTA.	Brockmann *et al* (3)
Sensitivity	>80% excellent, 70-80% good, 60-69% fair, <60% poor	No consensus in this regard exists in the literature.
Specificity	>90% excellent, 80-90% good, 70-79% fair, <70% poor	No consensus in this regard exists in the literature.
***Youden's* Index**	*Youden's* Index values close to 1 indicate high accuracy; a value of zero is equivalent to uninformed guessing and indicates that a test has no diagnostic value.	Macaskill *et al* (4)

DOR: diagnostic odds ratio; LR: likelihood ratio; DTA**:** diagnostic test accuracy

References

1. De Luca Canto G, Pachêco-Pereira C, Aydinoz S, Major PW, Flores-Mir C, Gozal D. Diagnostic Capability of Biological Markers in Assessment of Obstructive Sleep Apnea: A Systematic Review and

Meta-Analysis. J Clin Sleep Med. 2015;11:27-36. doi:10.5664/jcsm.4358.

2. Glas AS, Lijmer JG, Prins MH, Bonsel GJ, Bossuyt PMM. The diagnostic odd ratio: a single inidicator of test performance. J Clin Epidemiol. 2003;56:1129-35. doi:10.1016/S085-4356(03)00177-X.
3. Brockmann PE, Schaefer C, Poets A, Poets CF, Urschitz MS. Diagnosis of obstructive sleep apnea in children: a systematic review. Sleep Med Rev 2013; 17:331-40. doi:10.1016/j.smrv.2012.08.004.
4. Macaskill P, Gatsonics C, Deeks JJ, Harbord RM, Takwoingi Y. Cochrane Handbook for Systematic Review of Diagnostic Test Accuracy Version 1.0. In: Deeks JJ BP, Gatsonis C ed. Cochrane Handbook for Systematic Reviews of Diagnostic Test Accuracy Version 1.0. City: Cochrane, 2010:61.

Supplementary Table 3. Excluded articles and reasons for exclusion (n=15).

Author, Year	Reason for exclusion
Cavaco *et al*., 2018(1)	1
DeFelice *et al*., 2019(2)	2
Grimaldi *et al*., 2015(3)	3
He *et al*., 2012(4)	4
Hsiao *et al*., 2017(5)	5
Jinno, *et al*.,2015(6)	6
Koc *et al*., 1996(7)	7
Li *et al*., 2012(8)	5
Rekha *et al*.,2016(9)	5
Shigeyama *et al*., 2018(10)	1
Sugimoto, *et al*.,2011(11)	7
Tankiewcz *et al*., 2016(12)	2
Tran *et al*., 2015(13)	2
Yan, *et al*., 2008(14)	5
Yuvaraj *et al*., 2014(15)	5

1-studies with volatile metabolites;
2-studies that did not have healthy control group;
3-studies that included tumors located in salivary glands;
4-studies not written in English,
5- studies that did not evaluate or made an identification of the salivary metabolites;
6-studies that were not primary research articles, including reviews, letters, personal opinions, book chapters, and conference abstracts;

7- full text were not available

REFERENCES

1. Cavaco C, Pereira JAM, Taunk K, Taware R, Rapole S, Nagarajaram H, et al. Screening of salivary volatiles for putative breast cancer discrimination: an exploratory study involving geographically distant populations. Anal Bioanal Chem. 2018;410(18):4459-68.

2. DeFelice BC, Fiehn O. Rapid LC-MS/MS quantification of cancer related acetylated polyamines in human biofluids. Talanta. 2019;196:415-9.

3. Grimaldi M, Palisi A, Rossi G, Stillitano I, Faiella F, Montoro P, et al. Saliva of patients affected by salivary gland tumour: An NMR metabolomics analysis. J Pharm Biomed Anal. 2018;160:436-42.

4. He H, Shi X, Chen J, Gao P, Lei Y, Xu G. [Metabonomics study of oral cancer using gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry]. Se pu = Chinese journal of chromatography. 2012;30(3):245-51.

5. Hsiao YC, Chi LM, Chien KY, Chiang WF, Chen SF, Chuang YN, et al. Development of a Multiplexed Assay for Oral Cancer Candidate Biomarkers Using Peptide Immunoaffinity Enrichment and Targeted Mass Spectrometry. Molecular & cellular proteomics : MCP. 2017;16(10):1829-49.

6. H. Jinno TM, M. Sunamura, M. Sugimoto, T. Hayashida, M. Takahashi, Y. Kitagawa, editor Identification of breast cancer-specific signatures in saliva metabolites using capillary electrophoresis mass spectrometry. ESMO 2014; 2014; Madrid, Spain2014.

7. Koç L, Yarat A, Emekli N, Serdengeçti S, Berkarda B. Salivary sialic acid and cancer. Journal of Marmara University Dental Faculty. 1996;2(2-3):523-6.

8. Li X, Yang T, Lin J. Spectral analysis of human saliva for detection of lung cancer using surface-enhanced Raman spectroscopy. J Biomed Opt. 2012;17(3):037003.

9. Pachaiappan R, Aruna P, Ganesan B, Koteeswaran D, Baludavid M, Ganesan S. Near infrared Raman spectroscopic characterization of blood plasma of normal, oral premalignant and malignant conditions - A pilot study. Journal of Raman Spectroscopy. 2015;46.

10. Shigeyama H, Wang T, Ichinose M, Ansai T, Lee SW. Identification of volatile metabolites in human saliva from patients with oral squamous cell carcinoma via zeolite-based thin-film microextraction coupled with GC-MS. (1873-376X (Electronic)).

11. Sugimoto M, Wong D, Soga T, Tomita M. Salivary metabolomics for oral cancer detection. Oral Cancer: Causes, Diagnosis and Treatment. 2011:271-8.

12. Tankiewicz A, Dziemianczyk D Fau - Buczko P, Buczko P Fau - Szarmach IJ, Szarmach Ij Fau - Grabowska SZ, Grabowska Sz Fau - Pawlak D, Pawlak D. Tryptophan and its metabolites in patients with oral squamous cell carcinoma: preliminary study. (1896-1126 (Print)).

13. TN T. Chemical Isotope Labeling LC-MS for Human Saliva and Urine Metabolomics. Alberta, Canada: University of Alberta; 2015.

14. Yan SK, Wei BJ, Lin ZY, Yang Y, Zhou ZT, Zhang WD. A metabonomic approach to the diagnosis of oral squamous cell carcinoma, oral lichen planus and oral leukoplakia. Oral oncology. 2008;44(5):477-83.

15. Yuvaraj M, Udayakumar K, Jayanth V, Prakasa Rao A, Bharanidharan G, Koteeswaran D, et al. Fluorescence spectroscopic characterization of salivary metabolites of oral cancer patients. Journal of photochemistry and photobiology B, Biology.2014;130:153-60.

Supplementary Table 4. Metabolites Super classes and Subclasses

HMDB ID	Metabolites	number of studies	Tumor Primary	super class (HMDB)	subclasse (HMDB)	Pathway	related pathway Ref	Ref.
HMDB0001881	1,2-propanediol	1	head and neck cancer, oral cancer	Organic oxygen compounds	Alcohols and polyols	Pyruvate Metabolism	https://www.genome.jp/kegg/pathway/map/hsa00620.html	(25)
HMDB0000115	glycolic acid	1	oral cancer	Organic acids and derivatives	Alpha hydroxy acids and derivatives	Glyoxylate and dicarboxylate metabolism;Carbon metabolism	https://www.genome.jp/dbget-bin/www_bget?C00160	(26)
HMDB0034301	Piperidine	1	breast cancer, oral cancer, pancreatic cancer	Organoheterocyclic compounds		Protein digestion and absorption	https://www.genome.jp/dbget-bin/www_bget?C01746	(29)
HMDB00	N1N12-	1	breast	Organic	Carboximidic	N/F	https://www.genome.jp/dbget-	(30)

02172	diacetyl-spermine		cancer	acids and derivatives	acids		bin/www_bget?cpd:C03413	
HMDB0041947	N1N8-diacetyl-spermidine	1	breast cancer	Organic acids and derivatives	Carboxylic acid derivatives	N/F		(30)
HMDB0000269	Sphinganine	1	oral cancer	Organic nitrogen compounds	Amines	Sphingolipid Metabolism	http://www.genome.jp/kegg/pathway/map/hsa00500.html	(32)
HMDB0000906	Trimethylamine	1	breast cancer, oral cancer, pancreatic cancer	Organic nitrogen compounds	Amines	Carbon metabolism	https://www.genome.jp/kegg/pathway/map/hsa01200.html	(29)
HMDB0000062	L-carnitine	1	oral cancer	Organic nitrogen compounds	Quaternary ammonium salts	Beta Oxidation of Very Long Chain Fatty Acids	https://www.genome.jp/kegg/pathway/map/hsa01040.html	(34)
HMDB0003357	N-Acetylornithine	1	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Arginine biosynthesis	https://www.genome.jp/kegg/pathway/map/hsa00220.html	(21)

HMDB0001325	N6,N6,N6-trimethyllysine	1	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Lysine degradation	https://www.genome.jp/dbget-bin/www_bget?C03793	(26)
HMDB0002273	4-hydroxy-L-glutamic acid	1	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Arginine and Proline Metabolism	https://www.genome.jp/kegg/pathway/map/hsa00330.html	(32)
HMDB0001149	5-Aminolevulinate	1	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Glycine and Serine Metabolism	https://www.genome.jp/dbget-bin/www_bget?C00430	(21)
HMDB0000056	Beta-Alanine	1	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	beta-Alanine metabolism	https://www.genome.jp/dbget-bin/www_bget?C00099	(29)
HMDB0000828	Carbamoylaspartate	1	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Alanine, aspartate and glutamate metabolism	https://www.genome.jp/dbget-bin/www_bget?C00438	(21)
HMDB0000192	Cystine	1	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Cysteine and methionine metabolism	https://www.genome.jp/dbget-bin/www_bget?C00491	(21)

HMDB00000125	Glutathione	1	Oral cancer pharyngeal cancer. Laryngeal cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Cysteine and methionine metabolism	https://www.genome.jp/dbget-bin/www_bget?C00051	(15)
HMDB00000742	homocysteine	1	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Cysteine and methionine metabolism	https://www.genome.jp/dbget-bin/www_bget?C00155	(35)
HMDB00000450	hydroxylisine	1	gastric cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Lysine degradation	https://www.genome.jp/dbget-bin/www_bget?C16741	(18)
HMDB00028932	leucine + isoleucine	1	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	amino acid degradation pathways	http://www.hmdb.ca/metabolites/HMDB0028932#physical_properties	(29)
HMDB00003357	N-Acetylornithine	1	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Arginine biosynthesis	https://www.genome.jp/dbget-bin/www_bget?C00437	(20)
HMDB00	N-nonanoylgly	1	oral cancer	Organic acids and	Amino acids, peptides, and	N/F		(32)

13279	cine			derivatives	analogues			
HMDB0001369	Pyrroline hydroxycarboxylic acid	1	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Arginine and Proline Metabolism	https://www.genome.jp/kegg/pathway/map/hsa00330.html	(29)
HMDB0029415	S-carboxymethyl-L-cysteine	1	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	N/F	https://www.genome.jp/dbget-bin/www_bget?cpd:C03727	(32)
HMDB0000650	Alpha-Aminobutyric acid	1	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	N/F	https://www.genome.jp/dbget-bin/www_bget?cpd:C02261	(29)
HMDB0002072	4-methoxyphenylacetic acid	1	oral cancer	Benzenoids	Anisoles	N/F		(35)
HMDB0002820	Methylimidazoleacetic	1	breast cancer, oral	Organoheterocyclic compound	Imidazoles	Histidine Metabolism	https://www.genome.jp/dbget-bin/www_bget?C05828	(29)

	acid		cancer, pancreatic cancer	s				
HMDB00000707	4-Hydroxyphenylpyruvic acid	1	breast cancer	Benzenoids	Phenylpyruvic acid derivatives	Phenylalanine and Tyrosine Metabolism	https://www.genome.jp/dbget-bin/www_bget?C01179	(37)
HMDB00029635	4-Methilbenzoate	1	oral cancer	Benzenoids	Benzoic acids and derivatives	Xylene degradation (não é rota HS)	https://www.genome.jp/dbget-bin/www_bget?C01454	(20)
HMDB00002428	terephthalic acid	1	oral cancer	Benzenoids	Benzoic acids and derivatives	Polycyclic aromatic hydrocarbon degradation (não é rota HS)	https://www.genome.jp/dbget-bin/www_bget?C06337	(26)
HMDB00001473	dihydroxyacetone phosphate	1	oral cancer	Organic oxygen compounds	Carbohydrates and carbohydrate conjugates	Fructose and Mannose Degradation	https://www.genome.jp/dbget-bin/www_bget?C00111	(21)
HMDB00000174	fucose	1	head and neck cancer,	Organic oxygen compound	Carbohydrates and carbohydrate conjugates	Fructose and Mannose Degradation	https://www.genome.jp/dbget-bin/www_bget?C01019	(25)

			oral cancer	s				
HMDB01112	glyceraldehyde 3-phosphate	1	oral cancer	Organic oxygen compounds	Carbohydrates and carbohydrate conjugates	Fructose and Mannose Degradation	https://www.genome.jp/dbget-bin/www_bget?C00118	(20)
HMDB00001548	ribose 5-phosphate	1	oral cancer	Organic oxygen compounds	Carbohydrates and carbohydrate conjugates	Pentose phosphate pathway	https://www.genome.jp/dbget-bin/www_bget?C00117	(21)
HMDB00000258	sucrose	1	glioblastoma	Organic oxygen compounds	Carbohydrates and carbohydrate conjugates	Starch and Sucrose Metabolism	https://www.genome.jp/dbget-bin/www_bget?cpd:C00089	(19)
HMDB00000122	glucose	1	oral cancer	Organic oxygen compounds	Carbohydrates and carbohydrate conjugates	Glycolysis;Galactose Metabolism;Gluconeogenesis	https://www.genome.jp/dbget-bin/www_bget?C00031	(23)
HMDB00002064	N1-acetylputrescine	1	breast cancer	Organic acids and derivatives	Carboximidic acids	Arginine and proline metabolism	https://www.genome.jp/dbget-bin/www_bget?C02714	(30)
HMDB00001186	N1-acetyl-spermine	1	breast cancer	Organic acids and	Carboxylic acid derivatives	N/F	https://www.genome.jp/dbget-bin/www_bget?cpd:C02567	(30)

				derivatives				
HMDB00000705	Hexanoylcarnitine	1	oral cancer	Lipids and lipid-like molecules	Fatty acid esters	Carnitine metabolism	journals.plos.org › plosgenetics › article › file › journa...	(32)
HMDB00002212	n-eicosanoic acid	1	oral cancer	Lipids and lipid-like molecules	Fatty acids and conjugates	Biosynthesis of unsaturated fatty acids	https://www.genome.jp/dbget-bin/www_bget?C06425	(35)
HMDB00000638	n-dodecanoic acid	1	oral cancer	Lipids and lipid-like molecules	Fatty acids and conjugates	Fatty acid biosynthesis	https://www.genome.jp/dbget-bin/www_bget?C02679	(35)
HMDB00000624	2-Hydroxy-4-methylpentanoate	1	oral cancer	Lipids and lipid-like molecules	Fatty acids and conjugates	N/F		(20,21)
HMDB00000666	heptanoic acid	1	oral cancer	Lipids and lipid-like molecules	Fatty acids and conjugates	N/F	https://www.genome.jp/dbget-bin/www_bget?cpd+C17714	(26)
HMDB00000806	n-Tetradecanoic acid	1	oral cancer	Lipids and lipid-like molecules	Fatty acids and conjugates	Fatty Acid Biosynthesis	https://www.genome.jp/dbget-bin/www_bget?C06424	(35)
HMDB00001085	leucotriene B4	1	head and neck	Lipids and lipid-like	Eicosanoids	Arachidonic acid metabolism	https://www.genome.jp/dbget-bin/www_bget?C02165	(24)

			cancer	molecules				
HMDB00 12273	palmitic amide	1	breast cancer	Lipids and lipid-like molecules	Fatty amides	Primary fatty acid amide metabolism	http://www.lipidmaps.org/data/LMSDRecord.php?&LMID=LMFA08010009	(37)
HMDB00 11530	MG (0:0/14:0/0:0)	1	breast cancer	Lipids and lipid-like molecules	Monoradylglycerol s	Glycerolipid metabolism	https://www.genome.jp/dbget-bin/www_bget?C01885	(37)
HMDB00 12344	PS (14:1/16:1)	1	breast cancer	Lipids and lipid-like molecules	Glycerophosphos erines	Phosphatidylcho line Biosynthesis PC(14:1(9Z)/16: 1(9Z))	http://www.hmdb.ca/metabolites/HMDB0012344#biolo gical_properties	(37)
HMDB00 10386	LysoPC (18:2)	1	breast cancer	Lipids and lipid-like molecules	Glycerophosphoc holines	arachidonate biosynthesis I	https://biocyc.org/compound?orgid=META&id=CPD-8347#tab=RXNS	(37)
HMDB00 10404	LysoPC (22:6)	1	breast cancer	Lipids and lipid-like molecules	Glycerophosphoc holines	Glycerophospho lipid metabolism	https://www.genome.jp/dbget-bin/www_bget?cpd:C04230	(37)
HMDB00 09498	PE (22:0/20:4)	1	breast cancer	Lipids and lipid-like molecules	Glycerophosphoet hanolamines	Phosphatidylcho line Biosynthesis PC(22:0/20:4(5Z ,8Z,11Z,14Z))	http://www.hmdb.ca/metabolites/HMDB0009498#biolo gical_properties	(37)

HMDB10382	LysoPC (16:0)	1	breast cancer	Lipids and lipid-like molecules	Glycerophosphocholines	Phospholipid Biosynthesis	http://www.genome.jp/kegg/pathway/map/hsa00564.html	(37)
HMDB0002815	LysoPC (18:1)	1	breast cancer	Lipids and lipid-like molecules	Glycerophosphocholines	Glycerolipid metabolism	https://www.genome.jp/dbget-bin/www_bget?cpd:C03916	(37)
HMDB0000086	Glycerophosphocholine	1	breast cancer, oral cancer, pancreatic cancer	Lipids and lipid-like molecules	Glycerophosphocholines	Glycerophospholipid metabolism; Retinol Metabolism	https://www.genome.jp/dbget-bin/www_bget?C00670	(29)
HMDB0011507	LysoPE (18:2/0:0)	1	breast cancer	Lipids and lipid-like molecules	Glycerophosphoethanolamines	Glycerophospholipid metabolism	https://www.genome.jp/dbget-bin/www_bget?C04438	(37)
HMDB0008100	PC (18:1/16:0)	1	breast cancer	Lipids and lipid-like molecules	Glycerophosphocholines	Phosphatidylcholine Biosynthesis PC(18:1(9Z)/16:0)	http://www.hmdb.ca/metabolites/HMDB0008100#taxonomy	(37)
N/D	Burimamide	1	breast cancer, oral cancer,	Organoheterocyclic compound	Imidazoles	Histamine H2/H3 receptor agonists/antago	https://www.genome.jp/dbget-bin/www_bget?C07448	(29)

			pancreatic cancer	s		nists		
HMDB00002302	3-indolepropionic acid	1	oral cancer	Organoheterocyclic compounds	Indolyl carboxylic acids and derivatives	microbial tryptophan metabolism (não é HS)	https://www.nature.com/articles/s41387-018-0046-9	(35)
HMDB00000050	Adenosine	1	oral cancer	Nucleosides, nucleotides, and analogues	-	Purine metabolism;Selenoamino Acid Metabolism	https://www.genome.jp/dbget-bin/www_bget?cpd:C00212	(21)
HMDB00000195	Inosine	1	oral cancer	Nucleosides, nucleotides, and analogues	-	Purine Metabolism	https://www.genome.jp/dbget-bin/www_bget?C00294	(21)
HMDB00000008	2-hydroxybutyrate	1	Pancreatic cancer	Organic acids and derivatives	Alpha hydroxy acids and derivatives	Propanoate Metabolism; Malonic Aciduria;	http://www.hmdb.ca/metabolites/HMDB0000008#biological_properties	(16)
HMDB00000019	2-oxoisovaleric	1	oral cancer	Organic acids and	Short-chain keto acids and	Valine, Leucine and Isoleucine	http://www.hmdb.ca/metabolites/HMDB0000019#biological_properties	(26)

	acid			derivatives	derivatives	Degradation		
HMDB0000042	acetic acid	1	glioblastoma	Organic acids and derivatives	Carboxylic acids	Pyruvate Metabolism	http://www.hmdb.ca/metabolites/HMDB0000042#biological_properties	(19)
HMDB0000094	Citrate	1	oral cancer	Organic acids and derivatives	Tricarboxylic acids and derivatives	Citrate cycle (TCA cycle);Alanine, aspartate and glutamate metabolism	https://www.genome.jp/dbget-bin/www_bget?C00158	(21)
HMDB0000965	Hypotaurine	1	oral cancer	Organic acids and derivatives	Sulfinic acids	Taurine and Hypotaurine Metabolism	http://www.hmdb.ca/metabolites/HMDB0000965#biological_properties	(21)
HMDB0000005	2-oxobutyrate	1	Pancreatic cancer	Organic acids and derivatives	Short-chain keto acids and derivatives	Methionine Metabolism; Glycine and Serine Metabolism; Congenital lactic acidosis	http://www.hmdb.ca/metabolites/HMDB0000005#biological_properties	(16)
HMDB0000744	Malate	1	oral cancer	Organic acids and	Beta hydroxy acids and	Malate-Aspartate Shuttle;Glucone	https://www.genome.jp/dbget-bin/www_bget?C00149	(21)

				derivatives	derivatives	ogenesis; Citrate cycle (TCA cycle); Pyruvate metabolism		
HMDB00237	propionate	1	glioblastoma	Organic acids and derivatives	Carboxylic acids	Propanoate metabolism;Carbohydrate digestion and absorption;Nicotinate and nicotinamide metabolism	https://www.genome.jp/dbget-bin/www_bget?C00163	(19)
HMDB00062	carnitine	1	oral cancer	Organic nitrogen compounds	Quaternary ammonium salts	Lysine degradation;Carnitine Synthesis;Beta Oxidation of Very Long Chain Fatty Acids;Oxidation of Branched Chain Fatty Acids	https://www.genome.jp/dbget-bin/www_bget?C00487	(32)

HMDB0000020	p-hydroxyphenylacetic acid	1	oral cancer	Benzenoids	1-hydroxy-2-unsubstituted benzenoids	Tyrosine metabolism; Phenylalanine metabolism	https://www.genome.jp/dbget-bin/www_bget?cpd:C00642	(26)
HMDB0000755	Hydroxyphenyllactic acid	1	oral cancer	Phenylpropanoids and polyketides		tyrosine metabolism	http://www.hmdb.ca/metabolites/HMDB0000755#biological_properties	(32)
N/D	Piperideine	1	breast cancer, oral cancer, pancreatic cancer	Organoheterocyclic compounds		Propane, piperidine and pyridine alkaloid biosynthesis;	https://www.genome.jp/dbget-bin/www_bget?C06181	(29)
HMDB0001488	Nicotinate	1	oral cancer	Organoheterocyclic compounds	Pyridinecarboxylic acids and derivatives	Nicotinate and Nicotinamide Metabolism;Tropane, piperidine and pyridine alkaloid biosynthesis	https://www.genome.jp/dbget-bin/www_bget?C00253	(21)
HMDB0000469	5-hydroxymethyluracil	1	oral cancer	Organoheterocyclic compound	Pyrimidines and pyrimidine derivatives	DNA metabolism	https://books.google.com.br/books?id=aCP6-fd2i20C&pg=PA149&lpg=PA149&dq=5-hydroxymethyluracil+Metabolic+Pathways&source=bl&ots=hvHLesxYT-	(32)

				s			&sig=ACfU3U20NKiXBGrmMnVC0EN3GxLLUrpsSA&hl=pt-BR&sa=X&ved=2ahUKEwii3cSdvsbkAhU6IbkGHcV_DyMQ6AEwCHoECAoQAQ#v=onepage&q=5-hydroxymethyluracil%20Metabolic%20Pathways&f=false	
HMDB00000630	cytosine	1	oral cancer	Organoheterocyclic compounds	Pyrimidines and pyrimidine derivatives	Pyrimidine metabolism	https://www.genome.jp/dbget-bin/www_bget?C00380	(21)
HMDB00013305	Propionylcholine	1	oral cancer	Organic nitrogen compounds	Quaternary ammonium salts			(32)
HMDB00000149	ethanolamine	2	breast cancer, gastric cancer, glioblastoma, oral cancer, pancreatic cancer	Organic nitrogen compounds	Amines	Phospholipid Biosynthesis	http://www.genome.jp/kegg/pathway/map/hsa00564.html	(18, 19)

HMDB0004610	Phytosphingosine	2	oral cancer; breast cancer	Organic nitrogen compounds	Amines	Sphingolipid metabolism	https://www.genome.jp/dbget-bin/www_bget?C12144	(32) (37)
HMDB0001414	putrescine	2	breast cancer, oral cancer, pancreatic cancer	Organic nitrogen compounds	Amines	Methionine Metabolism	https://www.genome.jp/kegg/pathway/map/hsa00270.html	(30, 29)
HMDB0001257	Spermidine	2	breast cancer, oral cancer	Organic nitrogen compounds	Amines	Methionine Metabolism	https://www.genome.jp/kegg/pathway/map/hsa00270.html	(20,30)
HMDB0000206	N-ε-Acetyllysine	2	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Lysine degradation	https://www.genome.jp/dbget-bin/www_bget?C02727	(20,21)
HMDB0000272	O-Phosphoserine	2	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Glycine, serine and threonine metabolism	https://www.genome.jp/dbget-bin/www_bget?C01005	(20) (21)
HMDB0000452	2-aminobutyric	2	oral cancer	Organic acids and	Amino acids, peptides, and	Cysteine and methionine	https://www.genome.jp/dbget-bin/www_bget?C02356	(20 ,21)

	acid			derivatives	analogues	metabolism		
HMDB00000512	Acetylphenylalanine	2	breast cancer, oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Phenylalanine metabolism	https://www.genome.jp/dbget-bin/www_bget?C03519	(32,37)
HMDB0028680	Alanylalanine	2	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	amino acid degradation pathways	http://www.hmdb.ca/metabolites/HMDB0028680#biological_properties	(20,21)
HMDB00000517	arginine	2	breast cancer, oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Urea Cycle	https://www.genome.jp/kegg/pathway/map/hsa00330.html	(38,27)
HMDB00000043	betaine	2	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Glycine, serine and threonine metabolism	https://www.genome.jp/dbget-bin/www_bget?C00719	(34,29)
HMDB00000112	gamma-Aminobutyric acid	2	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Glutamate Metabolism	http://www.genome.jp/kegg/pathway/map/hsa00250.html	(35)

HMDB00011733	Gly-Gly	2	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	N/F	https://www.genome.jp/dbget-bin/www_bget?cpd:C02037	(20,21)
HMDB00000759	Gly-Leu	2	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	N/F	https://www.genome.jp/dbget-bin/www_bget?cpd:C02155	(20,21)
HMDB00000092	N.N-Dimethylglycin	2	oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Glycine and Serine Metabolism	https://www.genome.jp/dbget-bin/www_bget?C01026	(20,21)
HMDB00012136	Isopropanolamine	2	oral cancer	Organic nitrogen compounds	Amines	N/F	https://www.genome.jp/dbget-bin/www_bget?cpd:C05771	(20,21)
HMDB00000925	Trimethylamine N-oxide	2	oral cancer	Organic nitrogen compounds	Aminoxides	N/F		(20,21)
HMDB00000124	fructose 6-phosphate	2	oral cancer	Organic oxygen compounds	Carbohydrates and carbohydrate conjugates	Galactose metabolism	https://www.genome.jp/dbget-bin/www_bget?C00085	(20,21)
HMDB00	ribulose 5-	2	oral cancer	Organic	Carbohydrates	Pentose	https://www.genome.jp/dbget-	(20,21)

00618	phosphate			oxygen compounds	and carbohydrate conjugates	phosphate pathway;Lipopolysaccharide biosynthesis	bin/www_bget?cpd:C00199	
HMDB00000039	butyric acid	2	oral cancer	Lipids and lipid-like molecules	Fatty acids and conjugates	Butanoate metabolism; Carbohydrate digestion and absorption;Protein digestion and absorption	https://www.genome.jp/dbget-bin/www_bget?C00246	(26,20)
HMDB00000482	octanoic acid	2	oral cancer	Lipids and lipid-like molecules	Fatty acids and conjugates	Beta Oxidation of Very Long Chain Fatty Acids; Fatty acid biosynthesis	https://www.genome.jp/dbget-bin/www_bget?C06423	(26,20)
HMDB00000133	Guanosine	2	oral cancer	Nucleosides, nucleotides, and analogues	-	Purine metabolism	https://www.genome.jp/dbget-bin/www_bget?C00387	(20) (21)
HMDB00001185	S-adenosylmet	2	oral cancer	Nucleosides, nucleotides	5'-deoxy-5'-thionucleosides	Betaine Metabolism;Methionine	http://www.hmdb.ca/metabolites/HMDB0001185#biological_properties	(20)

	hionine			, and analogues		Metabolism		(21)
HMDB00000072	cis-Aconitate	2	oral cancer	Organic acids and derivatives	Tricarboxylic acids and derivatives	Citric Acid Cycle; Congenital lactic acidosis	http://www.hmdb.ca/metabolites/HMDB0000072#biological_properties	(20) (21)
HMDB00001276	N1 – acetylspermidine	2	breast cancer, pancreatic cancer	Organic acids and derivatives	Carboximidic acids	spermine and spermidine degradation I	https://biocyc.org/compound?orgid=META&id=CPD-568#tab=RXNS	(16,30)
HMDB00000254	Succinic acid	2	oral cancer	Organic acids and derivatives	Carboxylic acids	Citric Acid Cycle;Glutamate Metabolism;Mitochondrial Electron Transport Chain	https://www.genome.jp/dbget-bin/www_bget?C00042	(32,23)
HMDB00000294	urea	2	oral cancer	Organic acids and derivatives	Ureas	Urea Cycle;Arginine and Proline Metabolism;Purine metabolism	https://www.genome.jp/dbget-bin/www_bget?C00086	(20) (26)
HMDB00	7-Methylguani	2	oral cancer	Organoheterocyclic	Purines and	snRNA Cap Hypermethylatio	https://www.ebi.ac.uk/chebi/pathway.do?chebiId=CHE	(20,21)

00897	ne			compounds	purine derivatives	n; nuclear export of snRNA transcripts	BI:2274	
HMDB00132	guanine	2	oral cancer	Organoheterocyclic compounds	Purines and purine derivatives	Purine Metabolism	https://www.genome.jp/dbget-bin/www_bget?C00242	(20,21)
HMDB00289	uric acid	2	Oral cancer. pharyngeal cancer. Laryngeal cancer	Organoheterocyclic compounds	Purines and purine derivatives	Purine Metabolism	https://www.genome.jp/dbget-bin/www_bget?C00366	(15) (17)
HMDB00002	1,3-Diaminopropane	3	oral cancer; breast cancer	Organic nitrogen compounds	Amines	Beta-Alanine Metabolism	https://www.genome.jp/kegg/pathway/map/hsa00410.html	(20,21) (30)
HMDB00158	thyrosine	3	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Tyrosine Metabolism	http://www.genome.jp/kegg/pathway/map/hsa00350.html	(23,27,29)

HMDB0001161	γ-Butyrobetaine	3	oral cancer	Lipids and lipid-like molecules	Fatty acids and conjugates	Carnitine Synthesis	http://smpdb.ca/view/SMP00465?image_type=greyscale	(20,21)(26)
HMDB0000904	citrulline	3	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Arginine and Proline Metabolism	http://www.hmdb.ca/metabolites/HMDB0000904#biological_properties	(38,37,29)
HMDB0000696	methionine	3	breast cancer, oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Cysteine and methionine metabolism	https://www.genome.jp/dbget-bin/www_bget?cpd:C00073	(38,20,27)
HMDB0000187	serine	3	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Glycine, serine and threonine metabolism	https://www.genome.jp/dbget-bin/www_bget?C00065	(38,27,29)
HMDB0000535	hexanoic acid	3	oral cancer	Lipids and lipid-like molecules	Fatty acids and conjugates	Beta Oxidation of Very Long Chain Fatty Acids	https://www.genome.jp/kegg/pathway/map/hsa01040.html	(20,21,26)

HMDB00000665	2-Hydroxypentanoate	3	oral cancer	Lipids and lipid-like molecules	Fatty acids and conjugates	N/F		(20,21,26)
HMDB00000357	3-Hydroxybutyrate	3	oral cancer	Organic acids and derivatives	Beta hydroxy acids and derivatives	Fatty Acid Biosynthesis	http://www.hmdb.ca/metabolites/HMDB0000357#biological_properties	(20,21,26)
HMDB00000191	Aspartic acid	3	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Arginine and Proline Metabolism; aspartate Metabolism	http://www.hmdb.ca/metabolites/HMDB0000191#biological_properties	(38,27,29,21)
HMDB00000190	lactic acid	3	oral cancer	Organic acids and derivatives	Alpha hydroxy acids and derivatives	Gluconeogenesis;Pyruvate Metabolism; Fructose and mannose metabolism	https://www.genome.jp/dbget-bin/www_bget?C00186	(32,35,23)
HMDB00002189	N8-Acetylspermidine	3	breast cancer, oral cancer	Organic acids and derivatives	Carboximidic acids	N/F	https://www.genome.jp/dbget-bin/www_bget?cpd:C01029	(20,21,30)
HMDB00	3-phenyllactic	3	oral cancer	Phenylpropanoids and		Phenylalanine metabolism;Trop	https://www.genome.jp/dbget-bin/get_linkdb?-	(26)

00779	acid			polyketides		ane, piperidine and pyridine alkaloid biosynthesis	t+hmdb+cpd:C05607	
HMDB00000764	3-Phenylpropionate	3	oral cancer	Phenylpropanoids and polyketides		Phenylalanine metabolism	https://www.genome.jp/dbget-bin/www_bget?C05629	(20,21,26)
HMDB00000157	Hypoxanthine	3	breast cancer, oral cancer, pancreatic cancer	Organoheterocyclic compounds	Purines and purine derivatives	Purine Metabolism	https://www.genome.jp/dbget-bin/www_bget?C00262	(20,21,29)
HMDB00000123	glycine	4	breast cancer, gastric cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Glycine, serine and threonine metabolism	https://www.genome.jp/dbget-bin/www_bget?C00037	(18,23,27,29)
HMDB00	Pipecolate	4	breast cancer,	Organic acids and	Amino acids, peptides, and	Lysine	https://www.genome.jp/dbget-bin/www_bget?C00408	(20,21,3

00070			oral cancer, pancreatic cancer	derivatives	analogues	degradation		4,29)
HMDB00002199	3-(4-hydroxyphenyl)propionic acid	4	oral cancer	Phenylpropanoids and polyketides		tyrosine metabolism	http://www.hmdb.ca/metabolites/HMDB0002199#taxonomy	[(20,21,26)
HMDB00002322	cadaverine	5	breast cancer, oral cancer, pancreatic cancer	Organic nitrogen compounds	Amines	Lysine degradation	https://www.genome.jp/dbget-bin/www_bget?C01672	(20,21,26,29, 30)
HMDB00000641	glutamine	5	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Glutamate Metabolism	https://www.genome.jp/dbget-bin/www_bget?C00064	(18,21,23,29, 38)
HMDB00000177	histidine	5	breast cancer. gastric cancer.	Organic acids and derivatives	Amino acids, peptides, and analogues	Histidine metabolism	https://www.genome.jp/dbget-bin/www_bget?C00135	(18,27,37,29)

			oral cancer. pancreatic cancer					(38)
HMDB00000172	isoleucine	5	glioblastoma. Oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Valine, leucine and isoleucine biosynthesis	https://www.genome.jp/dbget-bin/www_bget?C00407	[19] [21] [23] [26] [27]
HMDB00000182	lysine	5	breast cancer, gastric cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Lysine biosynthesis	https://www.genome.jp/dbget-bin/www_bget?cpd:C00047	[18] [21] [27] [29] [38]
HMDB00000214	ornithine	5	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Arginine and proline metabolism	https://www.genome.jp/dbget-bin/www_bget?C00077	[38] [21] [30] [32] [29]
HMDB00000929	tryptophan	5	breast cancer, oral	Organoheterocyclic compound	Indolyl carboxylic acids and	Tryptophan metabolism,Phenylalanine,	https://www.genome.jp/dbget-bin/www_bget?C00078	[38] [20] [21] [26]

			cancer, pancreatic cancer	s	derivatives	tyrosine and tryptophan biosynthesis		[29]
HMDB00 00251	taurine	5	breast cancer, gastric cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Sulfinic acids	Taurine and Hypotaurine Metabolism;Bile Acid Biosynthesis	http://www.hmdb.ca/metabolites/HMDB0000251#biological_properties	[18] [20] [21] [26] [29]
HMDB00 00167	threonine	6	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Glycine and Serine Metabolism	http://www.genome.jp/kegg/pathway/map/hsa00260.html	[38] [20] [21] [27] [35] [29]
HMDB00 00148	glutamic acid	6	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Alanine, aspartate and glutamate metabolism	https://www.genome.jp/dbget-bin/www_bget?cpd:C00025	[38] [18] [23] [27] [29] [21]

HMDB0000159	phenylalanine	6	breast cancer, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Phenylalanine, tyrosine and tryptophan biosynthesis	https://www.genome.jp/dbget-bin/www_bget?C00079	[38] [27] [33] [35] [37] [29]
HMDB0000162	proline	6	breast cancer, gastric cancer, head and neck cancer, oral cancer, pancreatic cancer.	Organic acids and derivatives	Amino acids, peptides, and analogues	Arginine and proline metabolism	https://www.genome.jp/dbget-bin/www_bget?C00148	[38] [18] [23] [25] [35] [29]
HMDB0000097	choline	6	breast cancer, oral cancer, pancreatic cancer	Organic nitrogen compounds	Quaternary ammonium salts	Betaine Metabolism;Methionine Metabolism;Phospholipid Biosynthesis; Glycerophospho	https://www.genome.jp/dbget-bin/www_bget?C00114	[20] [21] [26] [34] [37] [29]

						lipid metabolism		
HMDB00000230	N-Acetylneuraminate	7	breast cancer, oral cancer; ovarian cancer	Organic oxygen compounds	Carbohydrates and carbohydrate conjugates	Amino sugar and nucleotide sugar metabolism	https://www.genome.jp/dbget-bin/www_bget?C00270	[21] [37] [14] [22] [28] [31] [36]
HMDB00000687	leucine	8	breast cancer, glioblastoma, oral cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Valine, leucine and isoleucine biosynthesis	https://www.genome.jp/dbget-bin/www_bget?C00123	[38] [19] [21] [23] [26] [27] [33] [35]
HMDB00000883	valine	9	breast cancer, glioblastoma, oral cancer, pancreatic cancer	Organic acids and derivatives	Amino acids, peptides, and analogues	Valine, leucine and isoleucine degradation	https://www.genome.jp/dbget-bin/www_bget?C00183	[38] [19] [20] [21] [23] [26] [27]. [35] [29]
HMDB00000161	alanine	11	Breast cancer, gastric	Organic acids and derivatives	Amino acids, peptides, and analogues	Alanine, aspartate and glutamate	https://www.genome.jp/dbget-bin/www_bget?C00041	[16] [38] [18] [19] [20] [21]

	cancer, glioblastoma, oral cancer, pancreatic cancer	metabolism	[23] [26] [27] [35] [29]

HMDB: Human Metabolome Database ;N/D: not described in HMDB ;N/F: not found in any database.

Suppplement Table 5. Super-classes and subclasses of reported metabolites

Organic oxygen compounds	**10**
Alcohols and polyols	1
Carbohydrates and carbohydrate conjugates	9

Lipids and lipid-like molecules	**23**
Fatty Acyls	13
Glycerolipids	1
Glycerophospholipids	9

Organic nitrogen compounds	**14**
Amines	9
Quaternary ammonium salts	4
Aminoxides	1

Benzenoids	**5**
Benzene and substituted derivatives	3
Phenol	1
Phenol ethers	1

Phenylpropanoic acids	**4**

Organoheterocyclic compounds	**13**
Piperidines	1
Azoles	2
Indoles and derivatives	2
Purines and purine derivatives	4
Pyridines and derivatives	2
Pyrimidines and pyrimidine derivatives	2

Organic acids and derivatives	**67**
Hydroxy acids and derivatives	5
Amino acids, peptides, and analogues	46
Keto acids and derivatives	2
Sulfinic acids and derivatives	2
Ureas	1
Carboximidic acids and derivatives	4
Others Carboxylic acid	7

derivatives

Nucleosides, nucleotides, and analogues	**4**
Purine nucleosides	3
5'-deoxyribonucleosides	1

Supplementary Table 6. Salivary metabolites for breast cancer.

Author	Sample Size (N)	BC (N)	Controls (NON-BC) (N)	Prevalence (%) #	Metabolite	Sensitivity (%)	Specificity (%)	PPV (%) #	NPV (%) #	LR+ #	LR- #	DOR #	Younden's Index Value #
Takayama *et al.* 2016 (30)	172	61	111	35	Spermine	74	70	57	83	2.46	0.37	6.65	0.44
Takayama *et al*. 2016(30)	172	61	111	35	N1-acetyl-spermidine	62	82	64	80	3.44	0.46	7.48	0.44
Takayama *et al*. 2016(30)	172	61	111	35	N8-acetyl-spermidine	65	77	60	80	2.82	0.45	6.27	0.42
Takayama *et al*. 2016(30)	172	61	111	35	N1-acetylputrescine	65	75	58	79	2.6	0.46	5.65	0.4
Takayama *et al*. 2016(30)	172	61	111	35	Cadaverine	67	73	57	80	2.48	0.45	5.51	0.4

Takayama *et al*. 2016(30)	172	61	111	35	Putrescine	67	72	56	80	2.39	0.45	5.31	0.39
Takayama *et al*. 2016(30)	172	61	111	35	N1-acetyl-spermine	62	73	55	78	2.29	0.52	4.4	0.35
Takayama *et al*. 2016(30)	172	61	111	35	Spermidine	68	65	51	79	1.94	0.49	3.96	0.33
Takayama *et al*. 2016(30)	172	61	111	35	Ornithine	70	59	47	78	1.7	0.5	3.4	0.29
Takayama *et al*. 2016(30)	172	61	111	35	N1N8-diacetyl-spermidine	72	50	43	76	1.44	0.56	2.57	0.22
Takayama *et al*. 2016(30)	172	61	111	35	N1N12-diacetyl-spermine	52	65	44	71	1.48	0.73	2.03	0.17
Takayama *et al*. 2016(30)	172	61	111	35	Diaminopropane	51	50	35	65	1.02	0.98	1.04	0.01
Takayama *et al*.	172	61	111	35	N8-acetyl-spermidine; N1-acetyl-spermidine,	80	79	67	88	3.8	0.25	15.2	0.59

2016(30)					Cadaverine, N1N12-diacetyl-spermine, Putrescine, and N1-acetylputrescine								
Zhong *et al*. 2016 (37)	55	30	25	54	LysoPC (18:2)	84	92	92	83	10.5	0.17	61.76	0.76
Zhong *et al*. 2016(37)	55	30	25	54	Palmitic amide	65	77	76	05	2.82	0.45	6.27	0.42
Zhong *et al*. 2016(37)	55	30	25	54	Phytosphingosine	80	92	92	79	10	0.21	47.62	0.72
Zhong *et al*. 2016(37)	5	30	25	54	LysoPC (18:1)	77	100	100	78	infinity	0.23	#VALUE!	0.77
Zhong *et al*. 2016(37)	55	30	25	54	PS (14:1/16:1)	77	91	90	77	8.55	0.25	34.2	0.68
Zhong *et al*. 2016(37)	55	30	25	54	LysoPC (16:0)	48	95	91	60	9.6	0.54	17.78	0.43

Zhong *et al*. 2016(37)	55	30	25	54	Acetylphenylalanine	80	74	78	75	3.07	0.27	11.37	0.54
Zhong *et al*. 2016(37)	55	30	25	54	Propionylcholine	53	85	80	60	3.53	0.55	6.42	0.38
Zhong *et al*. 2016(37)	55	30	25	54	LysoPC (22:6)	81	91	91	80	9	0.2	45	0.72
Zhong *et al*. 2016(37)	55	30	25	54	MG (0:0/14:0/0:0)	92	91	92	90	10.22	0.08	127.75	0.83
Zhong *et al*. 2016(37)	55	30	25	54	LysoPE (18:2/0:0)	92	62	73	86	2.42	0.12	20.17	0.54
Zhong *et al*. 2016(37)	55	30	25	54	PC (18:1/16:0)	59	91	88	65	6.55	0.45	14.56	0.5
Zhong *et al*. 2016(37)	55	30	25	54	Phenylalanine	77	66	72	70	2.26	0.34	6.65	0.43
Zhong *et al*.	55	30	25	54	Citruline	96	54	71	92	2.08	0.07	29.71	0.5

2016(37)												
Zhong *et al*. 2016(37)	55	30	25	54	Histidine	96	62	74	92	2.62	0.06	43.67	0.58
Zhong *et al*. 2016(37)	55	30	25	54	N-acetylneuraminic acid	92	58	72	86	2.19	0.13	16.85	0.5
Zhong *et al*. 2016(37)	55	30	25	54	PE (22:0/20:4)	70	75	76	68	2.8	0.4	7	0.45
Cheng *et al*. 2015 (38)	55	27	28	49	Pro, Thr, His	88	85	85	88	6.0	0.14	43.92	0.73
Cheng *et al*. 2015(38)	45	17 (stage I +II)	28	37	Phenylalanine	64	81	66	79	3.36	0.44	7.64	0.45
Cheng *et al*. 2015(38)	38	10 (stage III+IV)	28	26	Phenylalanine	70	82	57	88	3.88	0.36	10.78	0.52
Cheng *et*	45	17	28	37	Tryptophan	82	71	62	87	2.88	0.24	12.00	0.53

al. 2015(38)		(stage I +II)											
Cheng *et al*. 2015(38)	38	10 (stage III+IV)	28	26	Tryptophan	90	71	52	95	3.10	0.14	22.14	0.61
Cheng *et al*. 2015(38)	45	17 (stage I +II)	28	37	Methionine	82	71	62	87	2.88	0.24	12.00	0.53
Cheng *et al*. 2015(38)	38	10 (stage III+IV)	28	26	Methionine	90	71	52	95	3.14	0.14	22.43	0.61
Cheng *et al*. 2015(38)	45	17 (stage I +II)	28	37	Proline	70	92	83	83	8.75	0.32	27.34	0.62
Cheng *et al*. 2015(38)	38	10 (stage III+IV)	28	26	Proline	80	92	77	92	11.26	0.21	53.62	0.72
Cheng *et al*.	45	17 (stage	28	37	Threonine	76	85	74	85	5.06	0.28	18.07	0.61

2015(38)		I +II)											
Cheng *et al*. 2015(38)	38	10 (stage III+IV)	28	26	Threonine	90	85	67	96	6.00	0.11	54.55	0.75
Cheng *et al*. 2015(38)	45	17 (stage I +II)	28	37	Aspartic acid	82	67	59	86	2.48	0.26	9.54	0.49
Cheng *et al*. 2015(38)	38	10 (stage III+IV)	28	26	Aspartic acid	80	67	45	90	2.42	0.29	8.34	0.47
Cheng *et al*. 2015(38)	45	17 (stage I +II)	28	37	Serine	76	67	57	82	2.30	0.35	6.57	0.43
Cheng *et al*. 2015(38)	38	10 (stage III+IV)	28	26	Serine	90	71	52	95	3.10	0.14	22.14	0.61
Cheng *et al*. 2015(38)	45	17 (stage I +II)	28	37	Citruline	82	64	57	85	2.27	0.28	8.11	0.46

Cheng *et al*. 2015(38)	38	10 (stage III+IV)	28	26	Citruline	80	71	49	90	2.75	0.28	9.82	0.51
Cheng *et al*. 2015(38)	45	17 (stage I +II)	28	37	Ornithine	70	71	58	80	2.41	0.42	5.74	0.41
Cheng *et al*. 2015(38)	38	10 (stage III+IV)	28	26	Ornithine	70	85	62	88	4.66	0.35	13.31	0.55
Cheng *et al*. 2015(38)	45	17 (stage I +II)	28	37	Histidine	52	82	62	74	2.88	0.58	4.97	0.34
Cheng *et al*. 2015	38	10 (stage III+IV)	28	26	Histidine	50	82	49	82	2.77	0.60	4.62	0.32
Cheng *et al*. 2015(38)	45	17 (stage I +II)	28	37	Glutamine	58	82	65	76	3.22	0.51	6.31	0.40
Cheng *et al*.	38	10	28	26	Glutamine	90	64	46	94	2.50	0.15	16.67	0.54

2015(38)		(stage III+IV)										
Cheng *et al*. 2015(38)	45	17 (stage I +II)	28	37	Leucine	76	75	64	84	3.04	0.32	9.50	0.51
Cheng *et al*. 2015(38)	38	10 (stage III+IV)	28	26	Leucine	100	71	54	100	3.44	0.00	#DIV/0!	0.71
Cheng *et al*. 2015(38)	45	17 (stage I +II)	28	37	Valine	70	71	58	80	2.41	0.42	5.74	0.41
Cheng *et al*. 2015(38)	38	10 (stage III+IV)	28	26	Valine	90	92	79	96	11.25	0.10	112.50	0.82
Cheng *et al*. 2015(38)	45	17 (stage I +II)	28	37	Glutamic acid	58	89	75	78	5.27	0.47	11.21	0.47
Cheng *et al*. 2015(38)	38	10 (stage	28	26	Glutamic acid	90	89	74	96	8.18	0.11	74.36	0.79

		III+IV)											
Cheng *et al*. 2015(38)	45	17 (stage I +II)	28	37	Lysine	76	60	52	80	1.90	0.40	4.75	0.36
Cheng *et al*. 2015(38)	38	10 (stage III+IV)	28	26	Lysine	80	51	36	87	1.63	0.39	4.18	0.31

Data not available in the original article. The authors calculated data from information available in the article.

BC = breast cancer. PPV = positive predictive value. NPV = negative predictive value. LR+ = positive likelihood ratio. LR- = negative likelihood ratio. DOR = diagnostic odds ratio. Pro = Proline. Thr = Threonine. His = Histidine.

Supplementary Table 7. Salivary metabolites for oral cancer

Author	Sample Size (N)	OC (N)	Controls (NON-OC) (N)	Prevalence (%) #	Metabolites	Sensitivity (%)	Specificity (%)	PPV (%) #	NPV (%) #	LR+ #	LR- #	DOR #	Younden's Index Value #
Wang, 2014, China (34)	43	Stage I-II (13)	30	30	Choline	84	90	78	92	8.40	0.18	47.25	0.74
Wang *et al*., 2014, China (34)	43	Stage I-II (13)	30	30	Betaine	46	96	83	80	11.5	0.56	20.44	0.42
Wang *et al*., 2014, China (34)	43	Stage I-II (13)	30	30	Pipecolinic acid	92	96	90	96	23.0	0.08	276,00	0,88
Wang *et al*., 2014,	43	Stage I-II	30	30	L-carnitine	73	61	44	84	1,87	0,44	4,23	0,34

China (34)		(13)											
Wang *et al*., 2014, China (34)	47	Stage III-IV (17)	30	36	Choline	82	96	92	90	20,5	0,19	109,33	0,78
Wang *et al*., 2014, China (34)	47	Stage III-IV (17)	30	36	Betaine	47	80	56	72	2.35	0.66	3.55	0.27
Wang *et al*., 2014, China (34)	47	Stage III-IV (17)	30	36	Pipecolinic acid	82	96	92	90	20.5	0.19	109.33	0.78
Wang *et al*., 2014, China (34)	47	Stage III-IV (17)	30	36	L-carnitine	96	52	52	95	2.00	0.08	26.00	0.48
Wang *et al*., 2014, China (33)	73	Stage I-II (13)	60	18	L-phenylalanine	84	61	30	94	2.15	0.26	8.21	0.45

Wang *et al*., 2014, China (33)	73	Stage I-II (13)	60	18	L-leucine	84	81	47	96	4.42	0.2	22.38	0.65
Wang *et al*., 2014, China (33)	77	Stage III-IV (17)	60	22	L-phenylalanine	47	95	72	86	9.4	0.56	16.85	0.42
Wang *et al*., 2014, China (33)	77	Stage III-IV (17)	60	22	L-leucine	82	80	53	94	4.1	0.23	18.22	0.62
Wang *et al*., 2014, China (32)	43	Stage I-II (13)	30	30	Lactic acid	46	93	73	80	6.57	0.58	11.32	0.39
Wang *et al*., 2014, China (32)	43	Stage I-II (13)	30	30	Hydroxyphenyllactic acid	92	46	42	93	1.7	0.17	9.8	0.38
Wang *et al*., 2014,	43	Stage I-II	30	30	N-nonanoylglycine	69	73	52	84	2.56	0.42	6.02	0.42

China (32)		(13)											
Wang *et al*., 2014, China (32)	43	Stage I-II (13)	30	30	5-hydroxymethyluracil	53	96	85	82	13.2	0.49	27.06	0.49
Wang *et al*., 2014, China (32)	43	Stage I-II (13)	30	30	Succinic acid	69	83	63	86	4.06	0.37	10.87	0.52
Wang *et al*., 2014, China (32)	43	Stage I-II (13)	30	30	Ornithine	53	86	61	81	3.79	0.55	6.93	0.39
Wang *et al*., 2014, China (32)	43	Stage I-II (13)	30	30	Hexaoylcarnitine	61	83	60	83	3.59	0.47	7.64	0.44
Wang *et al*., 2014, China (32)	43	Stage I-II (13)	30	30	Propionylcholine	76	96	89	90	19	0.25	76	0.72

Wang *et al*., 2014, China (32)	43	Stage I-II (13)	30	30	Carnitine	92	50	44	93	1.84	0.16	11.5	0.42
Wang *et al*. 2014, China (32)	43	Stage I-II (13)	30	30	4-hydroxy-L-glutamic acid	92	50	44	93	1.84	0.16	11.5	0.42
Wang *et al*. 2014, China (32)	43	Stage I-II (13)	30	30	Acetylphenylalanine	92	76	62	95	3.83	0.11	36.42	0.68
Wang *et al*., 2014, China (32)	43	Stage I-II (13)	30	30	Sphinganine	84	83	67	92	4.94	0.19	25.63	0.67
Wang *et al*., 2014, China (32)	43	Stage I-II (13)	30	30	Phytosphingosine	92	83	69	96	5.41	0.1	56.15	0.75
Wang *et al*., 2014,	43	Stage I-II	30	30	S-carboxymethyl-L-cysteine	84	93	83	93	12	0.17	69.75	0.77

China (32)		(13)											
Wang *et al*., 2014, China (32)	47	Stage III-IV (17)	30	36	Lactic acid	100	73	67	100	3.7	0	#DIV/0!	0.73
Wang *et al*., 2014, China (32)	47	Stage III-IV (17)	30	36	Hydroxyphenyllactic acid	82	60	53	85	2.05	0.3	6.83	0.42
Wang *et al*., 2014, China (32)	47	Stage III-IV (17)	30	36	N-nonanoylglycine	52	73	52	73	1.93	0.66	2.93	0.25
Wang *et al*., 2014, China (32)	47	Stage III-IV (17)	30	36	5-hydroxymethyluracil	47	96	86	76	11.7	0.55	21.28	0.43
Wang *et al*., 2014, China (32)	47	Stage III-IV (17)	30	36	Succinic acid	88	66	59	90	2.59	0.18	14.24	0.54

Wang *et al*., 2014, China (32)	47	Stage III-IV (17)	30	36	Ornithine	82	73	63	87	3.04	0.25	12.32	0.55
Wang *et al*., 2014, China (32)	47	Stage III-IV (17)	30	36	Hexaoylcarnitine	70	60	49	78	1.75	0.5	3.5	0.3
Wang *et al*., 2014, China (32)	47	Stage III-IV (17)	30	36	Propionylcholine	64	80	64	79	3.2	0.45	7.11	0.44
Wang *et al*., 2014, China (32)	47	Stage III-IV (17)	30	36	Carnitine	94	46	49	93	1.74	0.13	13.35	0.4
Wang *et al*., 2014, China (32)	47	Stage III-IV (17)	30	36	4-hydroxy-L-glutamic acid	94	56	54	94	2.14	0.11	19.94	0.5
Wang *et al*., 2014,	47	Stage III-IV	30	36	Acetylphenylalanine	82	70	60	87	2.73	0.26	10.63	0.52

China (32)		(17)											
Wang *et al*., 2014, China (32)	47	Stage III-IV (17)	30	36	Sphinganine	70	83	69	83	4.12	0.36	11.39	4.12
Wang *et al*., 2014, China (32)	47	Stage III-IV (17)	30	36	Phytosphingosine	76	83	71	86	4.47	0.29	15.46	4.47
Wang *et al*., 2014, China (32)	47	Stage III-IV (17)	30	36	S-carboxymethyl-L-cysteine	88	90	83	93	8.8	0.13	66	8.8
Vajaria *et al*. 2013 (31)	200	100	100	50	Total sialic acid, Total protein	61	44	52	53	1.08	0.88	1.23	0.05
Wei *et al*. 2011 (35)	71	37	34	52	Lactic acid and Phenylalanine	94	82	84	92	5.22	0.07	74.57	0.76

Wang *et al*. 2014 China (34)	60	Stage I-II (30)	30	50	Choline, Betaine, Pipecolinic acid, L-carnitine	100	97	97	100	33.3	0	NC	0.97
Wang *et al*. 2014 China (34)	60	Stage III-IV (30)	30	50	Choline, Betaine, Pipecolinic acid, L-carnitine	88	90	89	88	8.80	0.13	66.00	0.78
Wang *et al*. 2014 China (33)	90	Stage I-II (30)	60	33	L-phenylalanine and L-leucine	92	81	70	95	4.84	0.10	49.03	0.73

Wang *et al*. 2014 China (33)	90	Stage III-IV (30)	60	33	L-phenylalanine and L-leucine	94	75	64	96	3.76	0.08	47.00	0.69
Wang *et al*., 2014 (32)	60	Stage I-II (30)	30	50	Propionylcholine, N-AcetylpL-phenylalanine, Sphingainne, Phytosphingosine, S-Carboxymethyl-L-cysteine	100	96	96	100	25.00	0.00	#DIV/0!	0.96
Wang *et al*., 2014 (32)(3	60	Stage III-IV (30)	30	50	Propionylcholine, N-AcetylpL-phenylalanine, Sphingainne, Phytosphingosine, S-Carboxymethyl-L-cysteine	86	94	93	87	14,33	0.15	96.24	0.80

Data not available in the original article. The authors calculated data from information available in the article.

OC = oral cancer. PPV = positive predictive value. NPV = negative predictive value. LR+ = positive likelihood ratio. LR- = negative likelihood ratio. DOR = diagnostic odds ratio.

Supplementary Table 8. DTA of salivary metabolites of tumors from other locations

Author	Cancer type	Sample size (N)	Cancer (N)	Controls (Non-cancer)	Prevalence (%) #	Metabolite	Sensitivity (%)	Specificity (%)	PPV (%) #	NPV (%) #	LR+ #	LR- #	DOR #	Younden's Index Value #
Mikkonen *et al*, 2018, Finland (25)	OC, Laryngeal cancer	75	45	30	0.6	Flucose, Glycin, Methanol and Proline	0.87	0.93	0.94	0.82	12.42	0.13	95.54	0.8
Chen *et al*, 2018, China(18)	Advanced gastric cancer	200	84	116	0.42	Taurine, Glycine, Glutamine, Ethanolamine, Histidine, Alanine, Glutamic acid, Hydroxylysine, Proline, Tyrosine	0.84	0.87	0.82	0.88	6.46	0.18	35.13	0.71

Chen *et al,* 2018, China(18)	Early gastric cancer	136	20	116	0.15	Taurine, Glycine, Glutamine, Ethanolamine, Histidine	0.8	0.88	0.54	0.96	6.67	0.23	29.33	0.68
Chen *et al*, 2018, China (18)	Ovarian cancer	52	15	37	0.15	Taurine, Glycine, Glutamine, Ethanolamine, Histidine	0.8	1	1	0.92	Infinity	0.2	#VALUE!	0.68

Data not available in the original article. The authors calculated data from information available in the article.

HNC = head and neck cancer. PPV = positive predictive value. NPV = negative predictive value. LR+ = positive likelihood ratio. LR- = negative likelihood ratio. DOR = diagnostic odds ratio.

	Item	Achalli, 2017, India	Almadori, 2007, Italy	Asai, 2018, Japan	Bahar, 2007, Israel	Chen , 2018 , China	Garcia, 2018, Italy	Ishikawa, 2016, Japan	Ishikawa, 2017, Japan	Jacob 2016, India
Domain 1: Patient Selection	Was a consecutive or random sample of patients enrolled?	U	Y	U	U	U	U	U	U	Y
	Was a case-control design avoided?	N	N	N	N	N	N	N	N	N
	Did the study avoid inappropriate exclusions?	Y	N	N	N	Y	N	N	N	N
	Could the selection of patients have introduced bias?	H	H	H	H	H	H	H	H	H
	Concerns regarding applicability: Is there concern that the included patients do not match the review question?	L	L	L	L	L	L	L	L	L
Domain 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard?	U	U	U	U	U	U	U	U	U
	If a threshold was used, was it pre-specified?	N	N	N	N	N	N	N	N	N
	Could the conduct or interpretation of the index test have introduced bias?	U	U	U	U	U	U	U	U	U
	Concerns regarding applicability: Is there concern that the index test, its conduct, or interpretation differ from the review question?	L	L	L	L	L	L	L	L	L
Domain 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Y	Y	Y	U	U	U	U	U	U
	Were the reference standard results interpreted without knowledge of the results of the index test?	U	U	U	U	U	U	U	U	U
	Could the reference standard, its conduct, or its interpretation have introduced bias?	U	U	U	U	U	U	U	U	U
	Concerns regarding applicability: Is there concern that the target condition as defined by the reference standard does not match the review question?	L	L	L	U	U	U	U	U	U
Domain 4: Flow and Timing	Was there an appropriate interval between index test(s) and reference standard?	U	U	U	U	U	U	U	U	U
	Did all patients receive a reference standard?	Y	Y	Y	U	U	U	U	U	U
	Did patients receive the same reference standard?	Y	Y	Y	U	U	U	U	U	U
	Were all patients included in the analysis?	Y	Y	Y	Y	Y	Y	Y	Y	U
	Could the patient flow have introduced bias?	U	U	U	U	U	U	U	U	U

Li, 2012, China	Lohavanichbut, 2018, USA	Malone, 1994, USA	Mikkonen, 2018, Filand	Ohshima, 2017, Japan	Reddy, 2012, India	Sanjay, 2008, India	Sugimoto, 2010, Japan	Takayama 2016, Japan
U	U	U	Y	U	U	U	U	U
N	N	N	N	N	N	N	N	N
N	N	N	N	Y	Y	Y	Y	Y
H	H	H	H	H	H	H	H	H
L	L	L	L	L	L	L	L	L
U	U	U	U	U	U	U	U	U
N	N	N	N	N	N	N	N	N
U	U	U	U	U	U	U	U	U
L	L	L	L	L	L	L	L	L
U	U	U	U	U	Y	Y	U	U
U	U	U	U	U	U	U	U	U
U	U	U	U	U	U	U	U	U
U	U	U	U	U	U	U	U	U
U	U	U	U	U	U	U	U	U
U	U	U	U	U	Y	Y	U	U
U	U	U	U	U	Y	Y	U	U
U	Y	Y	Y	Y	Y	Y	U	U
U	U	U	U	U	U	U	U	U

	Item	Vajaria, 2013, India	Wang 2014 China (88)	Wang 2014 China (Talanta)	Wang, 2014 Scientific reports	Wei, 2011,China	Zermeno-Nava, 2018 Mexico	Zhong 2016 China
Domain 1: Patient Selection	Was a consecutive or random sample of patients enrolled?	U	U	U	U	U	Y	U
	Was a case-control design avoided?	N	N	N	N	N	Y	N
	Did the study avoid inappropriate exclusions?	Y	Y	Y	Y	Y	Y	Y
	Could the selection of patients have introduced bias?	H	H	H	H	H	L	H
	Concerns regarding applicability: Is there concern that the included patients do not match the review question?	L	L	L	L	L	L	L
Domain 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard?	U	U	U	U	U	U	U
	If a threshold was used, was it pre-specified?	N	N	N	N	N	N	N
	Could the conduct or interpretation of the index test have introduced bias?	U	U	U	U	U	U	U
	Concerns regarding applicability: Is there concern that the index test, its conduct, or interpretation differ from the review question?	L	L	L	L	L	L	L
Domain 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Y	Y	Y	Y	Y	Y	Y
	Were the reference standard results interpreted without knowledge of the results of the index test?	U	U	U	U	U	U	U
	Could the reference standard, its conduct, or its interpretation have introduced bias?	U	U	U	U	U	U	Y
	Concerns regarding applicability: Is there concern that the target condition as defined by the reference standard does not match the review question?	L	L	L	L	L	L	L
Domain 4: Flow and Timing	Was there an appropriate interval between index test(s) and reference standard?	U	U	U	U	U	U	U
	Did all patients receive a reference standard?	Y	Y	Y	Y	Y	Y	Y
	Did patients receive the same reference standard?	Y	Y	Y	Y	Y	Y	Y
	Were all patients included in the analysis?	Y	Y	Y	Y	Y	Y	Y
	Could the patient flow have introduced bias?	U	U	U	U	U	U	U

Supplementary Table 9. QUADAS 2 complete list of questions and answers

2.3 Article 3: Salivary metabolite profiles in breast cancer patients determined using untargeted metabolomics

Daniele Xavier Assad [1,2]

Ana Carolina Acevedo Poppe [1]

Elisa Cançado Porto Mascarenhas [1, 3]

Ana Gabriela Costa Normando [1]

Valérie Pichon [4,5]

Helene Chardin [4,6]

Eliete Neves Silva Guerra [1]

Audrey Combes [4,5*]

[1] Laboratory of Oral Histopathology, Health Sciences Faculty, University of Brasília

Campus Universitário Darcy Ribeiro, CEP: 70910-900, Brasília DF, Brazil

[2] Medical Oncology Department, Hospital Sírio-Libanês, SGAS 613 Conj. E Bl. B, Brasília DF, Brazil

[3] Medical Oncology Department, Cettro- Centro de Câncer de Brasília, SMH/N Quadra 02, 12º Andar, Brasilia, DF, Brazil.

[4] Department of Analytical, Bioanalytical Sciences and Miniaturization (LSABM), ESPCI Paris, UMR CBI 8231, PSL Research University, Paris, France

[5] Sorbonne Université, Campus UPMC, Paris, France

[6] Faculté de Chirurgie Dentaire, Université Paris Descartes Sorbonne Paris Cité, Paris, France

ABSTRACT

The potential use of metabolomics in the oncology research field is been evaluated in many reports. This study aimed to explore whether salivary metabolites could help discriminate between breast cancer (BC) patients and healthy control subjects. Saliva samples from 23 BC patients and 35 healthy controls were subjected to untargeted metabolomics using ultra performance liquid chromatography-quadrupole time-of-flight mass spectrometry and an online bioinformatics tool (XCMS Online), which revealed 534 molecular features that were shared by the two groups. The METLIN database was searched for 31 ions that were significantly up-regulated in the BC group ($p<0.05$), which identified 7 oligopeptides and 6 glycerophospholipids (PG14:2, PA32:1, PS28:0, PS40:6, PI31:1, and PI38:7). In addition, saliva samples were evaluated from before and after treatment of 10 patients who experienced at least partial treatment response. In these patients, 3 peptides and PG14:2 were up-regulated before treatment but not after treatment. The lipids' area under the curve (AUC) values ranged from 0.5875 (PI31:1) to 0.7329 (PG14:2), the sensitivity values ranged from 43.48% (PI31:1) to 65.22% (PG14:2), and the specificity values ranged from 57.14% (PS40:6) to 88.57% (PS28:0). These results provide new information regarding the salivary metabolite profiles of BC patients, which may be useful biomarkers. However, further validation of these results in needed in a larger cohort of BC patients and healthy controls.

Keywords: breast cancer, metabolomics, XCMS, METLIN, mass spectometry

1. INTRODUCTION

Metabolic alteration is a hallmark of cancer cells and their malignant transformation is characterized by multiple changes in metabolic pathways that are linked to macromolecule synthesis(1,2). Thus, cancer cells have altered metabolic requirements to facilitate inappropriate replication and survival, and these cells must simultaneously coordinate nutrient uptake and metabolism to meet their catabolic and anabolic demands. The classical example of a reprogrammed metabolic pathway in cancer is the Warburg effect or “aerobic glycolysis”(3).

Breast cancer (BC) is the most commonly diagnosed cancer and the leading cause of cancer-related mortality among women.4 The diagnosis of BC has two main steps, which involve identification of a suspected lesion via radiological screening and a confirmatory biopsy, as conventional screening with physical examination and mammography provides less-than-desirable sensitivity (54%) and specificity (77%) (5,6). Breast biopsy and histopathology studies are the reference standard for diagnosis, although these techniques are invasive and involve a risk of morbidity (7). Therefore, research has focused on identifying biomarkers as a potential adjunctive tool for diagnosing BC. In this context, saliva is a fluid that reflects the body’s physiological condition, and has recently emerged as a biological fluid that could be used to monitor clinical status and identify systemic diseases (8). While saliva is 99% water, it also contains mucus, electrolytes (K^+ ions at 25 mmol/L and Na^+ ions at 2 mmol/L), nucleic acids, and proteins(8,9). Relative to blood-based testing, saliva-based testing is simpler, easier, and safer, given the non-invasive collection methods(10). In 2017, Sugimoto *et al.* described salivary metabolic profiles for oral, breast, and pancreatic cancers based on an untargeted metabolomic approach using capillary electrophoresis time-of-flight mass spectrometry, which identified 14 amino acids (AAs, including taurine and lysine) that were significantly elevated in BC patients relative to in healthy controls(11). In addition, a review of salivary biomarkers for diagnosing BC revealed that sialic acid, taurine, proline, and valine provided potential diagnostic value(12).

Recent development of bioinformatic tools has made it relatively simple to automate the identification of distinct metabolite features from different groups of samples and patients(13). For example, the XCMS Online tool detects and identifies chromatographic features with varying relative intensity values for comparison between sample groups, with the reported data including the p-values and fold changes(14,15). These tools have potential utility for identifying early subclinical markers that can be used to predict the development of BC and facilitate early intervention. Therefore, the present study used an untargeted metabolomic approach to identify salivary metabolites that were differentially expressed in BC patients and healthy controls, with comparisons of the two groups' saliva profiles performed using the XCMS Online tool and identification performed using the METLIN database.

2. MATERIALS AND METHODS

Ethical considerations

This prospective study was conducted in accordance with the principals to the Declaration of Helsinki and the study protocol was approved by the Research Ethics Committee of the Faculty of Health Sciences, University of Brasilia (UnB, DF, Brazil; Plataforma Brasil protocol: 57449716.5.0000.0030). All individuals provided written informed consent before participating in the study.

Study participants

The control subjects were healthy women who were recruited from the general population to undergo a normal physical examination and radiological breast imaging. Consecutive BC patients were recruited from oncology centers at the *Hospital Universitário de Brasília*, *Hospital de Base do Distrito Federal*, *Hospital Sírio-Libanês,* and *Centro de Câncer de Brasília-Cettro* between October 2016 and October 2017. The inclusion criteria for the BC patient group were: i) not pregnant or lactating; ii) no active oral/dental disease; iii) no prior neoplasia, except for non-melanomatous skin cancers, cervical carcinoma in situ, or benign tumors (e.g., adenomas); iv) no impaired

renal function, congestive heart failure, or active infection (e.g., hepatitis and HIV); and v) a histopathological diagnosis of BC. These patients were enrolled before any systemic treatment (neo-adjuvant chemotherapy or palliative endocrine/chemotherapy) and before definitive surgery for excision of the breast tumor.

There was no central pathology review for the present study. Tumor staging was performed using the TNM system from the 7th edition of the American Joint Committee on Cancer guidelines(16). Molecular profile classification was performed according to the Saint Gallen consensus: i) luminal A-like: *all of* estrogen receptor (ER)-positive, progesterone receptor (PR)-positive, human epidermal growth factor receptor 2 (HER2)-negative, and Ki-67 'low'; ii) luminal B-like HER2-negative: ER-positive, HER2-negative, and *at least one of* Ki-67 'high' and PR-negative or PR-low; iii) luminal B-like HER2-positive: ER-positive, HER2 over-expressed or amplified, any Ki-67, and any PR; iv) HER2-positive (non-luminal): HER2 over-expressed or amplified, ER-negative, and PR-negative; v) triple-negative: ER-negative, PR-negative, and HER2-negative(17). Stage I–III cases were treated using neo-adjuvant chemotherapy with or without double HER2 blockade, followed by surgery with or without radiotherapy and with or without adjuvant endocrine treatment. Stage IV cases were treated using palliative chemotherapy or endocrine therapy with or without double HER2 blockade.

Specimen collection, transportation, and preparation

Stimulated saliva samples were collected from each participant, who abstained from eating, drinking, smoking and performing oral hygiene procedures for ≥1 h before the collection. At the collection time, the participants were instructed to chew a cotton swab (Salivette®; Sarstedt AG & Co., Nümbrecht, Oberbergischer Kreis, Germany) for 2 min. The swab was then placed in a plastic container and packaged in a Styrofoam box with recyclable ice packets for <4 h before being transported to the laboratory for processing. The saliva sample (typically 5–10 mL) was centrifuged for 5 min at 3,000 rpm and 8°C. After centrifugation, the sample was transferred to a

clean Eppendorf tube and frozen at –80°C until the analysis. Before the liquid chromatography (LC) and mass spectrometry (MS), the saliva samples were diluted with one volume of the mobile phase used for LC separation (90:10, water with 0.1% formic acid (v/v)/acetonitrile with 0.1% formic acid, v/v). High-performance liquid chromatography (HPLC)-grade acetonitrile and formic acid were supplied by CarloErba (Val de Reuil, France), and ultra-pure water was obtained using a Milli-Q Purification system (Millipore, Molsheim, France).

Ultra high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF/MS)

The testing was performed using an Agilent 1100 LC system (Agilent Technologies, Les Ulis, France) coupled to a MicrOTOF-Q II Mass Spectrometer (Bruker Daltonics, Wissem-bourg, France) with an electrospray ionization (ESI) source. The separation was performed using an Atlantis dC18 column at 40°C (150×2.1 mm, 3µm; Waters Corporation, Milford). The mobile phase was composed of (A) water with 0.1% formic acid (v/v) and (B) acetonitrile with 0.1% formic acid (v/v). The elution gradient started at 10% B, which was increased to 90% B over a 45-min interval, with a 2-min plateau, a 3-min return to the initial composition, and a 10-min final equilibration step. The flow-rate was set at 0.2 mL/min and the injection volume was 20 µL. The ESI-TOF-MS analysis was performed in the positive ionization mode, and the mass-detection range was set at m/z 50–1200. The ESI source parameters were a drying gas (N2) flowrate of 5.0 L/min, drying gas temperature of 200ºC, nebulizing gas pressure of 10 psi, and capillary voltage of 4500 V. The ion transfer method used two different settings: a couple collision RF/transfer time equal to (i) 100 Vpp/23 µs during 30% of the acquisition time (300 µs) and (ii) 400 Vpp/100 µs during 70% of the acquisition time (700 µs) (*i.e.*, an acquisition time of 1 s for each MS spectrum). The collision energy and the pre-pulse storage were maintained at 5 eV and 5 µs, respectively. All data acquisitions were controlled using TOF Control software (version 3.4, BrukerDaltonics), and Hystar software (version 3.2, Bruker Daltonics) was used to interface the UHPLC and MS systems.

Metabolic pathways

The metabolites identified using XCMS Online were used to search the Human Metabolome Database (hmdb.ca), the KEGG network (genome.jp), PubChem (pubchem.ncbi.nlm.nih.gov), the Small Molecule Pathway Database (smpdb.ca), and LIPID MAPS Lipidomics Gateway (lipidmaps.org).

Data analysis

The subjects' demographic and clinical characteristics were compared using Student's t test and the chi-squared or Fisher exact test as appropriate. Differences were considered statistically significant at p-values of <0.05. Receiver operating characteristic (ROC) curves were used to evaluate the predictive value of each biomarker, and were compared using the non-parametric method of DeLong *et al* (18). Optimal cut-off points on the ROC curves were identified based on i) the shortest Euclidean distance between the results of the binary classification test (100% sensitivity and 100% specificity) and ii) the maximum Euclidean distance between the results of the binary classification test (a 45º line). Sensitivity and specificity values, as well as the respective 95% confidence intervals, were calculated for each metabolite's optimal cut-off value. All statistical analyses were performed using SAS software (version 9.4).

The LC/MS profiles generated via UHPLC-Q-TOF/MS were converted into mzML files using Hystar software (Bruker Technology), which were then uploaded to XCMS Online (xcmsonline.scripps.edu) (19). All LC/MS profiles were processed using the "centerwave" algorithm with an allowance of 10 ppm on the experimental m/z and a minimum S/N ratio of 3 to extract the molecular features. The orbiwarp algorithm was used for the retention time correction, with a step size of 0.5 m/z and a maximum of 5 s allowed for deviation of the retention time.

3 RESULTS

Patient characteristics

The study included 23 women with BC (mean age: 47.52±9.79 years) and 35 healthy women from the general population (mean age: 42.00±13.83 years). The BC cases all involved invasive ductal carcinoma, with 1 case also involving a micropapillary component and 1 case involving squamous differentiation. None of the controls had a history of cancer treatment. Table 1 shows the demographic characteristics of the 50 subjects who provided saliva samples and the clinical characteristics of the 23 BC cases. There were no significant differences regarding age, menopause status, tobacco use, medication use, or childbearing. The complete subject characteristics are listed in Supplementary Table 1.

Table 1. Subject Charactheristics. A) Breast cancer and Healthy controls demographic data. B) Breast cancer cases clinical and pathological charactheristics.

A

Characteristic *	Breast cancers (n=23)	Healthy Controls (n=35)	p -value #
Mean age (range)- yr	47.52±9.79	42.00±13.83	0.1028
Menopause status			0.7072
Premenopause	14 (60.87)	23 (65.71)	
Menopause	9 (39.13)	12 (34.29)	
Tobacco use			0.2019
No	19 (82.61)	33 (94.29)	
Yes	4 (17.39)	2 (5.71)	
Use of medication			0.2446
No	16 (69.57)	19 (54.29)	
Yes	7 (30.43)	16 (45.71)	

Childbearing			0.2663
No	8 (36.36)	18 (51.43)	
Yes	14 (63.64)	17 (48.57)	

B

Grade	
Grade 1	2
Grade 2	13
Grade 3	8
Node status	
Node negative	13
Node positive	10
Stage	
Stage 1	2
Stage 2	12
Stage 3	5
Stage 4	4
ER status	
ER positivity ≥10%	14
ER positivity<10%	9
PR status	
PR positivity ≥10%	13
PR positivity <10%	10
HER2 status	
HER2 positive	11
HER2 negative	12

KI67 status	
KI67≤ 20%	9
KI67>20%	14
Molecular profile	
Luminal A-like	5
Luminal B HER2 negative- like	8
Luminal B HER2 positive- like	3
HER2 positive (non luminal)-like	3
Triple negative	3

yr: year. ER: estrogen receptor, PR : progesterone receptor, HER2: human epidermal growth factor receptor 2 , Ki67 : antigen KI-67 * Values expressed in median ± standard deviation or frequency (%). Additional baseline characteristics are listed in Supplementary Table 1 in the Supplementary Appendix. # p-value by Student's t test and chi-square/Fisher exact test

Quality control for the untargeted metabolomics

The conditions of the stored saliva samples were evaluated at different points throughout the –80ºC storage period, as well as after double injection of each sample. The results were similar at all time points, which indicated that the method was repeatable. The presence of previously described salivary metabolites in cancer patients (Supplementary Table 2) was manually verified in the samples, although we failed to detect significant inter-group differences (all p>0.05) (11,20-30).

The LC/MS profiles of the healthy controls and breast cancer patients

The LC/MS salivary profiles were compared between the BC patients and healthy controls using XCMS Online, which revealed 534 molecular features (same m/z and same retention) that were present in both groups. Among these 534 shared features, significant inter-group differences (up- or down-regulated, all p<0.05) were observed for 37 molecular features. The present study focused on up-regulated metabolites, and 1 ion was ignored because it was down-regulated in the BC patients. Thus, the chromatograms

for the 36 corresponding ions were manually evaluated, which confirmed that 35 ions were up-regulated and 1 ion was excluded because did not correspond to a chromatographic peak. The molecular weights of all known medications were compared to each up-regulated ion (according to charge state) in order to confirm that they were not prescribed medications or their metabolites (Supplementary Table 3). The proposed metabolite names were also screened using the METLIN database, which identified 4 of the 35 ions as potentially being drugs or phytochemical compounds. These compounds corresponded to dioscin (an antifungal agent), desglucomusennin (a phytochemical compound), donepezil (an oral medication used to treat Alzheimer's disease) and dilazep (a vasodilator). Tetrahydrogambogic acid was excluded because it is a compound isolated from fruits. (Table 2, lines 1–5).

Among the 31 remaining ions, the METLIN database proposed a putative identification for 13 metabolites (7 oligopeptides and 6 glycerophospholipids) based on the experimental m/z and charge state (Table 2, lines 5–17). Supplementary Figure 1 shows the chromatograms for these ions. The characteristics of the 18 unidentified up-related ions (LC retention, fold change between the patient and control groups, and p-value) are shown in Supplementary Table 4.

Table 2: Identified ions in Metlin database

	m/z	Retention time in HPLC	fold change (a)	p-value	Raw formula	Putative METLIN identification (b)	Mw g/mole (error in ppm)
1	380.22	3	1.8	0.03	$C_{24}H_{29}NO_3$	donazepil	379.2147 (1)
2	457.23	2.5	3.1	0.003	$C_{45}H_{72}O_{16}$	dioscin or desglucomusenin	868.482 (1)
3	622.33	3.1	2.3	0.04	$C_{32}H_{40}N_6O_6$	dilazep OR Thr-Trp-Trp- (Ile/Leu) OR Pro-Arg-Arg-Arg	604.3009 (1)
4	633.34	3.0	2.2	0.02	$C_{38}H_{48}O_8$	Tetrahydrogambogic acid	632.3349 (1)
5	440.23	3.4	3.7	0.03	$C_{15}H_{31}N_9O_5$	Arg-Arg-Ser	417.2448 (3)
6	442.24	3.1	2.1	0.01	$C_{18}H_{31}N_7O_6$	His-Lys-(Ala-Ser) or (Gly-Thr)	441.2335 (1)
7	543.23	54.8	2.2	0.03	$C_{26}H_{31}N_5O_5S$	Ala-Lys-Phe-Trp OR Gly-Lys-Thr-Ser OR Arg-Arg-Ser-Ser	525.2045 (1)
8	585.31	15.7	3.1	0.02	$C_{26}H_{42}N_8O_6$	Phe-Ile-Gln-Arg	562.3227 (2)
9	596.31	3.0	2.7	0.03	$C_{25}H_{38}N_8O_8$	Glu-Phe-Gln-Arg OR Ile-Lys-Gln-Trp	578.2812 (1)
10	630.33	3.1	1.9	0.04	$C_{32}H_{45}N_7O_5$	Phe-Lys-Lys-Trp or Phe-Gln-Arg-Tyr	607.3482 (1)
11	644.31	3.0	3.0	0.02	$C_{34}H_{38}N_6O_6$	Phe-Phe-Gln-Trp	626.2852 (1)
12	669.42	36.9	2.1	0.04	$C_{35}H_{67}O_7P$	PA 32:1 (c)	630.4624 (5)
13	718.39	19.4	6.3	0.01	$C_{34}H_{66}NO_{10}P$	PS 28:0 (c)	679.4423 (9)
14	817.46	2.4	3.5	0.04	$C_{40}H_{75}O_{12}P$	PI 31:1 (c)	778.4996 (9)
15	874.49	2.5	2.9	0.03	$C_{46}H_{78}NO_{10}P$	PS 40:6 (c)	835.5362 (7)
16	919.47	3.0	2.5	0.02	$C_{47}H_{77}O_{13}P$	PI 38:7 (c)	880.5101 (1)
17	533.22	2.5	2.4	0.01	$C_{23}H_{43}O_9P$	PG 14:2 (c)	494.2644 (1)

(a) Increasing fold change between the group of patients and of controls
(b) Metabolites putatively annotated according to results in METLIN database (add reference)
(c) The lipids and phospholipids were annotated according to the recommendation of Liebisch *et al* (journal of lipid research 2003)

Subgroup analyses of the LC/MS profiles

Subgroup analyses were performed according to the various molecular subgroupings of the BC samples (Ki-67: ≤20% vs. >20%, ER: ≥10% vs. <10%, Grade 1–2 vs. Grade 3, and HER2-positive vs. HER2-negative), as well as comparisons of the subgroups with the control group. The results of these subgroup analyses are shown in Figure 1. Table 3 shows the distributions of the 13 identified and up-regulated ions and its related pathways, which were not detected in the following subgroups: luminal B HER2-negative, HER2-positive, and triple-negative.

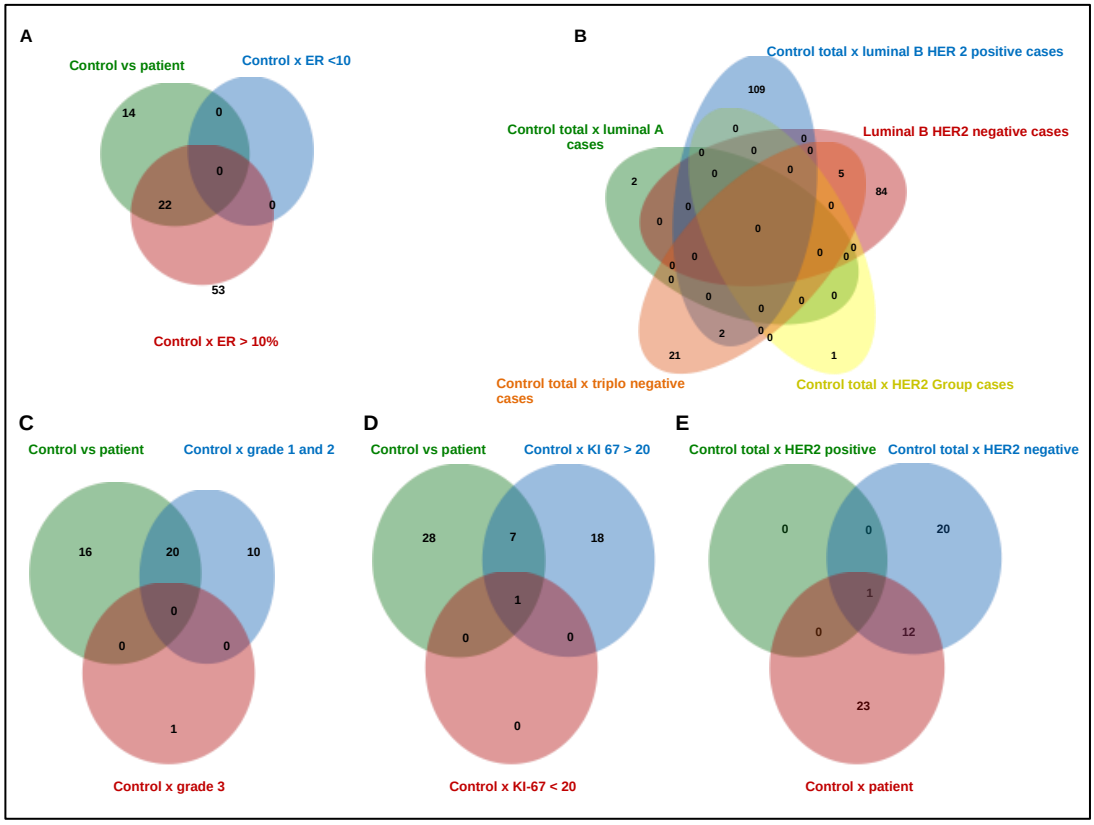


Figure 1: Venn Diagrams of subgroup analysis. A) Green circle Pairwise job of 35 HC vs 23 BC cases, Blue circle: Pairwise job of 35 HC vs 9 BC cases with ER<10%, Red circle:

Pairwise job of 35 HC vs 14 BC cases with ER> 10%. 22 ions present in ER>10% group. **B)** Green circle: Pairwise job of 35 HC vs 7 Luminal A BC cases, Blue circle: pairwise job of 35 HC vs 6 BC with Luminal B HER2 positive BC cases, Red circle: Pairwise job of 35 HC vs 2 Luminal B HER2 negative BC cases, Yellow circle: Pairwise job of 35 HC and 5 HER2 positive BC group, Orange circle: Pairwise job of 35 HC and 3 triple negative BC patients: 2 ions present in luminal A cases, 109 ions in luminal B HER2 positive cases, 84 in luminal B HER2 negative cases, 1 in HER2 group and 21 in triple negative cases. **C)** Green circle: Pairwise job of 35 HC vs 23 BC patients, Blue circle: Pairwise job of 35 HC vs 15 BC patients Grade 1 and grade 2, Red: Pairwise job of 35 HC vs 5 BC grade 3. 20 ions present in grade 1 & grade 2 group. **D)** Green circle Pairwise job of 35 HC vs 23 BC patients , Blue circle: Pairwise job in 35 HC vs 10 BC with Ki67>20%, Red circle: Pairwise job of 35 HC vs 13 BC with Ki 67 <20%: 7 ions present in Ki 67>20% group. E) Green circle: Pairwise job of 35 HC vs 23 BC patients, Blue circle: Pairwise job of 35 HC and 12 HER2 negative breast cancer cases, Red circle: Pairwise job of 35 HC and 11 HER2 positive breast cancer cases. 20 ions in HER2 negative BC cases.

Table 3: Subgroup analysis and pathways.

	METLIN identification	Related Pathway	ER≥1	Grade 1 & 2	Her2 negative	ki67>20	Luminal B HER2 +
440.23	Arg-Arg-Ser	N/S	Up	Up	up		up
442.24	His-Lys-(Ala-Ser) or (Gly-Thr)	N/S	up	up	up		
543.23	Ala-Lys-Phe-Trp or Gly-Lys-Thr-Ser or Arg-Arg-Ser-Ser	N/S	Up	up			
585.31	Phe-Ile-Gln-Arg	N/S	Up	up			
596.31	Glu-Phe-Gln-Arg or Ile-Lys-Gln-Trp	N/S					
630.33	Phe-Lys-Lys-Trp or Phe-Gln-Arg-Tyr	N/S	up				up
644.31	Phe-Phe-Gln-Trp	N/S					
		Triacylglycerol Biosynthesis					
669.42	PA 32:1 (c)	cardiolipin biosynthesis; glycerophospholipid metabolism	up	up			
718.39	PS 28:0 (c)	phosphatidylcholine biosynthesis, phosphatidylethanolamine biosynthesis, Glycerophospholipid metabolism and Lipid metabolism	up	up		up	

		pathway				
817.46	PI 31:1 (c)				up	up
874.49	PS 40:6 (c)	phosphatidylcholine biosynthesis, phosphatidylethanolamine biosynthesis, Glycerophospholipid metabolism and Lipid metabolism pathway				
919.47	PI 38:7 (c)	Lysolipid incorporation into ER pathway, phosphatidylcholine biosynthesis, phosphatidylethanolamine biosynthesis, Glycerophospholipid metabolism and Lipid metabolism pathway		up		
533.22	PG 14:2 (c)	glycerophospholipid metabolism	up	up	up	up

ER: estrogen receptor; N/S: not searched, PA: phosphatidic acid; PS: phosphatidylserine; PI : phosphatidylinositol, PG: phosphatidylglycerol

c- The lipids and phospholipids were annotated according to the recommendation of Liebisch *et al* (journal of lipid research 2003)

Comparing the LC/MS profiles before and after systemic treatments

Saliva samples were also evaluated from before and after the systemic treatment of 10 patients (8 cases involved neo-adjuvant treatment and 2 cases involved palliative treatment). All 10 patients experienced at least partial treatment response and none experienced disease progression. The patients' characteristics, treatments, and responses are listed in Supplementary Table 5. The control results were also compared to the patients' pre-treatment and post-treatment results (Figure 2). The 227 ions with altered pre-treatment regulation were searched for the 13 previously identified ions, which confirmed that 4 of the identified ions were up-related before treatment and were not up-related after treatment. These ions with pre-treatment up-regulation were m/z 440.23 (Arg-Arg-Ser), M/z 533.22 (PG14:2), m/z 543.23 (Ala-Lys-Phe-Trp *or* Gly-Lys-Thr-Ser *or* Arg-Arg-Ser-Ser) and m/z 630.33 (Phe-Lys-Lys-Trp *or* Phe-Gln-Arg-Tyr).

The ROC curve analysis

The ROC curves were analyzed to identify the optimal cut-off values for the salivary metabolites, and the sensitivity, specificity, and optimal cut-off values are listed in Table 4. Among the six identified lipids, the only significant difference in the AUC values was observed between PG14:2 and PA32:1 ($p=0.0434$). Supplementary Figure 2 shows the ROC curves for the lipids with the highest AUC values (PG14:2 and PI38:7).

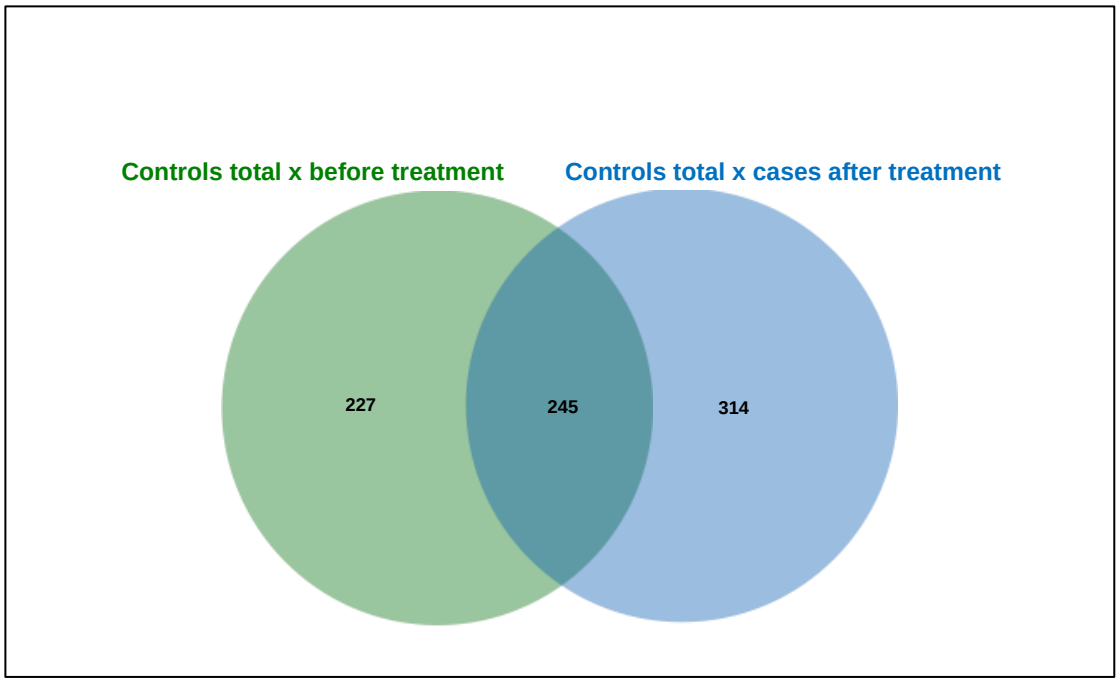


Figure 2: Venn Diagrams corresponding of comparison of LC/MS profiles before and after treatment. The green circle: all metabolites found in pairwise job of all 35 healthy controls vs 10 breast cancer patients before treatment. The blue circle represents all metabolites found in pairwise job of all 35 healthy controls vs 10 breast cancer patients after treatment. 245 ions in common among the “patients” in the group “before” and “after treatment” that did not change with the treatment. 227 ions were up or down regulated only before and were not present after treatment. 314 ions were only present after treatment.

Table 4: Values of the ROC curves analysis of lipids

Metabolites	AUC	CI 95%	Optimal cutoff value	Sensitivity	CI 95%	Specificity	CI 95 %
PG14:2	0.7329	0.5962-0.8697	19325.80	65.22%	42.77% – 83.62%	77.14%	59.86% - 89.58%
PA 32:1	0.5988	0.4319-0.7656	1915.30	60.87%	38.54% - 80.29%	60.00%	42.11%-76.23%
PS 28:0	0.6273	0.4644-0.7902	25012.90	47.83%	26.82%-69.41%	88.57%	73.26%-96.80%
PI 31:1	0.5876	0.4250-0.7502	37403.88	43.48%	23.19%-65.51%	82.86%	66.35%-93.44%
PS 40:6	0.5950	0.4357-0.7544	12784.24	56.52%	34.49%-76.81%	57.14%	39.35%-73.68%
PI 38:7	0.6609	0.5132-0.8085	1951.16	60.87%	38.54%-80.29%	71.43%	53.70%-85.36%

AUC: area under curve, CI: confidence interval.

4. DISCUSSION

The goal of metabolomics is to use a high-throughput system to identify and quantify endogenous and exogenous small-molecule metabolites in a biological system. The approach to metabolomic experiments can be untargeted or targeted, with targeted metabolomics (“biased or directed metabolomics” or “metabolic profiling”) focusing on either a pre-determined set of metabolites or a specific chemical class of small molecules (20,31,32). Untargeted metabolomics (“unbiased or undirected metabolomics” or “metabolic fingerprinting”) aims to detect as many metabolites as possible in a sample, in order to classify phenotypes based on metabolite patterns, which is

a hypothesis-generating approach that is well-suited for biomarker Discovery (11,13,33,34).The present study used an untargeted metabolomics approach to identify salivary metabolites that could help differentiate between BC patients and healthy controls.

Blood and saliva contain similar constituents, although saliva is a less expensive, simpler, and non-invasively collected diagnostic material.[35] Substances can pass through the epithelial membranes via several mechanisms, including a passive diffusion process (for highly lipid-soluble molecules), an active process against a concentration gradient (electrolytes, IgA), and ultrafiltration through membrane pores (small polar molecules with a molecular weight of <300 Da) (36). Based on these traits, we separated biomolecules in the patients' saliva using liquid chromatography (LC) and ionization, with MS to determine the m/z ratios of the ions that were derived by fragmenting the ionized parent compound. The full mass spectrums were then analyzed using a bioinformatics tool (XCMS Online), which identified differences in various key metabolites. We found that 31 ions were up-regulated in BC patients and potentially identified 13 ions, which included 7 oligopeptides and 6 lipids.

Tumor cells have dramatically altered AA uptake and secretion, relative to normal cells, which accounts for the majority of the carbon-based biomass production in rapidly proliferating cancer cells (37). In addition, AAs also contain nitrogen and are the dominant nitrogen source for hexosamines, nucleotides, and other nitrogenous compounds in rapidly proliferating cells(37). In this context, Cheng *et al*. used a targeted approach with UPLC-MS to evaluate specimens from 27 BC patients, and reported that the BC patients had higher salivary levels, relative to healthy controls, of arginine, ornithine, citrulline, alanine, methionine, glutamine, aspartic acid, phenylalanine, tryptophan, proline, threonine, serine, histidine, leucine, valine, glutamic acid, and lysine (38).

Sugimoto *et al*. have also used an untargeted metabolomics approach with CE-TOF-MS (87 healthy controls, 30 BC patients, 69 oral cancer patients, and 18 pancreatic cancer patients) (11). Their results identified 28 salivary

metabolites that were elevated in BC patients (all $p<0.05$), including 14 AAs with significantly altered values (e.g., taurine and lysine, $p<0.001$), although they did not perform direct identification. Nevertheless, there were no significant differences in these metabolites when the BC patients were compared to the patients with other cancers.

Zhong *et al*. used an untargeted approach with HPLC/MS (30 BC patients and 25 healthy controls) to identify 18 metabolites, including phenylalanine, citrulline, and histidine, which were confirmed using standard samples (34). The present study failed to detect significant differences in salivary AAs when we compared the BC patients and controls, which conflicts with the findings of Cheng *et al*., Zhong *et al*., and Sugimoto *et al*. However, the present study only included 23 cancer patients, while the previous studies included at least 30 patients, which might partially explain our conflicting findings. Furthermore, we used UHPLC-Q-TOF/MS, while the previous studies had used different analytical methods. Moreover, the present study evaluated Brazilian patients and the previous studies evaluated Asian patients, which suggests that ethnic differences might also have contributed to our conflicting findings (39,40). Given the lack of high-quality evidence, further research is needed to evaluate the relationship between salivary AAs and BC in larger and more diverse populations.

The *de novo* biosynthesis of fatty acids is low in normal adult tissues, although tumorigenesis is associated with a dramatic increase in lipid production, which has also been confirmed in BC patients (34,41). Phospholipids are an essential component of the cell membrane, and are involved in a variety of biological functions, such as division of the cytoplasm, inter-cell adhesion, and protein storage (42).

Based on our results, we identified six glycerophospholipids that might be related to BC: PG14:2, PA32:1, PS28:0, PS40:6, PI31:1, and PI38:7. The PG14:2 phosphatidylglycerol has a phosphoglycerol moiety occupying a glycerol substitution site, and is related to glycerophospholipid metabolism (43). The PA32:1 phosphatidic acid is a glycerophosphate with a phosphate moiety occupying a glycerol substitution site, and is extremely important as an

intermediate in the biosynthesis of triacylglycerols and phospholipids, which are related to the cardiolipin biosynthesis pathway(43,44).

Cardiolipin is an important component of the inner mitochondrial membrane, where it accounts for approximately 20% of the total lipid composition, and is essential for the optimal function of numerous enzymes that are involved in mitochondrial energy metabolism, as well as triacylglycerol biosynthesis (45,46).

The PS28:0 and PS40:6 phosphatidylserines have a phosphorylserine moiety occupying a glycerol substitution site, and are related to phosphatidylcholine biosynthesis, phosphatidylethanolamine biosynthesis, glycerophospholipid metabolism (45).The PI31:1 and PI38:7 phosphatidylinositols are key membrane constituents and participate in essential metabolic processes, both directly and via a number of metabolites (45). The PI38:7 phosphatidylinositol is related to lysolipid incorporation into to ER pathway, phosphatidylcholine biosynthesis, phosphatidylethanolamine biosynthesis and glycerophospholipid metabolism(45).

Zhong *et al*. used HPLC/MS analysis to identify salivary metabolites that were significantly elevated in BC patients (34). Their significant findings included glycerol phospholipid compounds (LysoPC18:2, LysoPC18:1, PS14:1/16:1, LysoPC16:0, LysoPC22:6, LysoPE18:2/0:0, PC18:1/16:0, and PE22:0/20:4), a fatty amide (palmitic amide), a sphingolipid (phytosphingosine), a choline (propionylcholine), and glyceroglycolipids (MG0:0/14:0/0:0). Our ROC curve analysis revealed no significant differences in the metabolites' areas under the curves, with the exception that PG14:2 was superior to PA32:1 (p=0.0434), and the highest AUC values were observed for PG14:2 (0.7329) and PI38:7 (0.6609).

Zhong *et al*. also identified high AUC values for predicting BC based on three up-regulated lipids, including LysoPC18:1 (AUC: 0.92), LysoPC22:6 (AUC: 0.92), and MG0:0/14:0/0:0 (AUC: 0.929) (34). These higher AUC values are clearly superior to the values for the lipids that we identified in the present study. However, the present study compared salivary compounds from before and after treatment in 10 patients who responded well to

treatment, and found that 4 ions (3 peptides and PG14:2) were up-regulated before treatment but subsequently normalized after treatment. While this subanalysis is clearly limited by the small sample size, the findings suggest that salivary biomarkers may be useful for monitoring treatment response, and we believe that ours is the first report to examine the effects on systemic cancer treatment on salivary metabolite profiles.

The main limitation of this study is the small number of BC patients. The lack of a standardized saliva collection time may also have influenced our findings regarding metabolite profiles, as Ishikawa *et al*. found that the fasting period can influence the metabolite profile for detecting oral cancer, with an optimal 12-h fast commencing after dinner (24). Therefore, additional experimentation and a more targeted analytical approach will be needed to validate the relevance of the metabolites that we identified.

In conclusion, the present study identified 31 up-regulated ions in BC patients, and we were able to potentially identify 13 metabolites via the METLIN database. These 13 ions included 7 peptides and 6 lipids (PG14:2, PA32:1, PS28:0, PS40:6, PI31:1, and PI38:7). Interestingly, a comparison of pre-treatment and post-treatment metabolite profiles from 10 patients revealed that 3 peptides and PG14:2 were up-regulated before treatment and returned to normal levels after treatment. Thus, while caution must be exercised when interpreting this finding, it is possible that these metabolites can be useful biomarkers for BC treatment response. The ROC curve analyses revealed that the salivary lipids provided good specificity and fair sensitivity for identifying BC, although a larger cohort of BC patients and healthy controls is needed to confirm our findings. Nevertheless, we believe that our results indicate that salivary testing may be useful for the early diagnosis of BC.

ACKNOWLEDGEMENTS

This work was supported by grants from *Fundação de Apoio à Pesquisa do Distrito Federal* (FAPDF, Brasília, Brazil) and DIM analytics from the *Ile de France* region. The authors declare no potential conflicts of interest regarding the authorship and/or publication of this report.

REFERENCES

1. Hanahan, D.; Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell*. **2011**, *144*, 646–674.

2. Dang, C. V. Links between metabolism and cancer. *Genes Dev*. **2012**, *26*, 877–890.

3. Lunt, S. Y.; Vander Heiden, M. G. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol*. **2011**, *27*, 441–464.

4. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R. L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. **2018**, *68*, 394–424.

5. Senkus, E.; Kyriakides, S.; Ohno, S.; Penault-Llorca, F.; Poortmans, P.; Rutgers, E.; et al. Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. **2015**, *26*, v8–v30.

6. Berg, W. A.; Gutierrez, L.; NessAiver, M. S.; Carter, W. B.; Bhargavan, M.; Lewis, R. S.; et al. Diagnostic accuracy of mammography, clinical examination, US, and MR imaging in preoperative assessment of. *Radiology* **2004**, *233*, 830–849.

7. Zhang, Y. J.; Wei, L.; Li, J.; Zheng, Y. Q.; Li, X. R. Status quo and development trend of breast biopsy technology. *Gland Surg* **2013**, *2*, 15–24.

8. de Almeida Pdel, V.; Gregio, A. M.; Machado, M. A.; de Lima, A. A.; Azevedo, L. R. Saliva composition and functions: a comprehensive review. *J Contemp Dent Pract*. **2008**, *9*, 72–80.

9. Chiappin, S.; Antonelli, G.; Gatti, R.; De Palo, E. F. Saliva specimen: a new laboratory tool for diagnostic and basic investigation. *Clin Chim Acta*. **2007**, *383*, 30–40.

10. Kaczor-Urbanowicz, K. E.; Martin Carreras-Presas, C.; Aro, K.; Tu, M.; Garcia-Godoy, F.; Wong, D. T. Saliva diagnostics – Current views and directions. *Exp Biol Med (Maywood).* **2017**, *242*, 459–472.

11. Sugimoto, M.; Wong, D. T.; Hirayama, A.; Soga, T.; Tomita, M. Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. *Metabolomics*. **2010**, *6*, 78–95.

12. Porto-Mascarenhas, E. C.; Assad, D. X.; Chardin, H.; Gozal, D.; De Luca Canto, G.; Acevedo, A. C.; et al. Salivary biomarkers in the diagnosis of breast cancer: A review. *Crit Rev Oncol Hematol*. **2017**, *110*, 62–73.

13. Patti, G. J.; Yanes, O.; Siuzdak, G. Innovation: Metabolomics: the apogee of the omics trilogy. *Nat Rev Mol Cell Biol*. **2012**, *13*, 263–269.

14. Johnson, C. H.; Ivanisevic, J.; Benton, H. P.; Siuzdak, G. Bioinformatics: the next frontier of metabolomics. *Anal Chem*. **2015**, *87*, 147–156.

15. Tautenhahn, R.; Patti, G. J.; Rinehart, D.; Siuzdak, G. XCMS Online: a web-based platform to process untargeted metabolomic data. *Anal Chem*. **2012**, *84*, 5035–5039.

16. American Joint Committee on Cancer. AJCC Cancer Staging Manual 2017. Available from: https://cancerstaging.org/Pages/default.aspx.

17. Goldhirsch, A.; Winer, E. P.; Coates, A. S.; Gelber, R. D.; Piccart-Gebhart, M.; Thurlimann, B.; et al. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert

Consensus on the Primary Therapy of Early Breast Cancer 2013. *Ann Oncol*. **2013**, *24*, 2206–2223.

18. DeLong, E.R.; DeLong, D.M.; Clarke-Pearson, D. L. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics*. **1988**, *44*, 837–845.

19. Benton, H. P.; Ivanisevic, J.; Mahieu, N. G.; Kurczy, M. E.; Johnson, C. H.; Franco, L.; et al. Autonomous metabolomics for rapid metabolite identification in global profiling. *Anal Chem*. 2015, 87, 884–891.

20. Asai, Y.; Itoi, T.; Sugimoto, M.; Sofuni, A.; Tsuchiya, T.; Tanaka, R.; et al. Elevated Polyamines in Saliva of Pancreatic Cancer. *Cancers (Basel)*. **2018**, *10*, E43.

21. Almadori, G.; Bussu, F.; Galli, J.; Limongelli, A.; Persichilli, S.; Zappacosta, B.; et al. Salivary glutathione and uric acid levels in patients with head and neck squamous cell carcinoma. *Head Neck*. **2007**, *29*, 648–654.

22. Chen, Y.; Cheng, S.; Zhang, A.; Song, J.; Chang, J.; Wang, K.; et al. Salivary Analysis Based on Surface Enhanced Raman Scattering Sensors Distinguishes Early and Advanced Gastric Cancer Patients from Healthy Persons. *J Biomed Nanotechnol*. **2018**, *14*, 1773–1784.

23. Ishikawa, S.; Sugimoto, M.; Kitabatake, K.; Sugano, A.; Nakamura, M.; Kaneko, M.; et al. Identification of salivary metabolomic biomarkers for oral cancer screening. *Sci Rep*. **2016**, *6*, 31520.

24. Ishikawa, S.; Sugimoto, M.; Kitabatake, K.; Tu, M.; Sugano, A.; Yamamori, I.; et al. Effect of timing of collection of salivary metabolomic biomarkers on oral cancer detection. *Amino Acids*. **2017**, *49*, 761–770.

25. Li, X.; Yang, T.; Lin, J. Spectral analysis of human saliva for detection of lung cancer using surface-enhanced Raman spectroscopy. *J Biomed Opt*. **2012**, *17*, 037003.

26. Ohshima, M.; Sugahara, K.; Kasahara, K.; Katakura, A. Metabolomic analysis of the saliva of Japanese patients with oral squamous cell carcinoma. *Oncol Rep*. **2017**, *37*, 2727–2734.

27. Reddy, I.; Sherlin, H. J.; Ramani, P.; Premkumar, P.; Natesan, A.; Chandrasekar, T. Amino acid profile of saliva from patients with oral squamous cell carcinoma using high performance liquid chromatography. *J Oral Sci*. **2012**, *54*, 279–283.

28. Takayama, T.; Tsutsui, H.; Shimizu, I.; Toyama, T.; Yoshimoto, N.; Endo, Y.; et al. Diagnostic approach to breast cancer patients based on target metabolomics in saliva by liquid chromatography with tandem mass spectrometry. *Clin Chim Acta*. **2016**, *452*, 18–26.

29. Wang, Q.; Gao, P.; Cheng, F.; Wang, X.; Duan, Y. Measurement of salivary metabolite biomarkers for early monitoring of oral cancer with ultra performance liquid chromatography-mass spectrometry. *Talanta*. **2014**, *119*, 299–305.

30. Wei, J.; Xie, G.; Zhou, Z.; Shi, P.; Qiu, Y.; Zheng, X.; et al. Salivary metabolite signatures of oral cancer and leukoplakia. *Int J Cancer*. **2011**, *129*, 2207–2217.

31. Achalli, S.; Madi, M.; Babu, S. G.; Shetty, S. R.; Kumari, S.; Bhat, S. Sialic acid as a biomarker of oral potentially malignant disorders and oral cancer. *Indian J Dent Res*. 2017, 28, 395–399.

32. Almadori, G.; Bussu, F.; Galli, J.; Limongelli, A.; Persichilli, S.; Zappacosta, B.; et al. Salivary glutathione and uric acid levels in patients with head and neck squamous cell carcinoma. *Head Neck*. **2007**, *29*, 648–654.

33. Garcia-Villaescusa, A.; Morales-Tatay, J. M.; Monleon-Salvado, D.; Gonzalez-Darder, J. M.; Bellot-Arcis, C.; Montiel-Company, J. M.; et al. Using NMR in saliva to identify possible biomarkers of glioblastoma and chronic periodontitis. *PLOS One*. **2018**, *13*, e0188710.

34. Zhong, L.; Cheng, F.; Lu, X.; Duan, Y.; Wang, X. Untargeted saliva metabonomics study of breast cancer based on ultra performance liquid

chromatography coupled to mass spectrometry with HILIC and RPLC separations. *Talanta*. **2016**, *158*, 351–360.

35. Roi, A.; Rusu, L. C.; Roi, C. I.; Luca, R. E.; Boia, S.; Munteanu, R. I. A New Approach for the Diagnosis of Systemic and Oral Diseases Based on Salivary Biomolecules. *Disease Markers*. **2019**, doi: 10.1155/2019/8761860.

36. Aps, J. K.; Martens, L. C. Review: The physiology of saliva and transfer of drugs into saliva. *Forensic Sci Int*. **2005**, *150*, 119–131.

37. Choi, B. H.; Coloff, J. L. The Diverse Functions of Non-Essential Amino Acids in Cancer. *Cancers (Basel)*. **2019**, *11*, E675.

38. Cheng, F.; Wang, Z.; Huang, Y.; Duan, Y.; Wang, X. Investigation of salivary free amino acid profile for early diagnosis of breast cancer with ultra performance liquid chromatography-mass spectrometry. *Clin Chim Acta*. **2015**, *447*, 23–31.

39. de Boer, J. O.; van Es, A. J.; Voorrips, L. E.; Blokstra, F.; Vogt, J. E. Energy metabolism and requirements in different ethnic groups. *Eur J Clin Nutr*. **1988**, *42*, 983–997.

40. O'Donnell, P. H.; Dolan, M. E. Cancer pharmacoethnicity: ethnic differences in susceptibility to the effects of chemotherapy. *Clin Cancer Res*. **2009**, *15*, 4806–4814.

41. Li, J.; Cheng, J. X. Direct visualization of de novo lipogenesis in single living cells. *Sci Rep*. 2014, 4, 6807.

42. Escribá, P. V.; González-Ros, J. M.; Goñi, F. M.; Kinnunen, P. K.; Vigh, L.; Sánchez-Magraner, L.; et al. Membranes: a meeting point for lipids, proteins and therapies. *J Cell Mol Med*. **2008**, *12*, 829–875.

43. Wishart, D. S.; Feunang, Y. D.; Marcu, A.; Guo, A. C.; Liang, K.; Vazquez-Fresno, R.; et al. HMDB 4.0: the human metabolome database for 2018. *Nucleic Acids Res*. **2018**, *46*, D608–D617.

44. Ejsing, C. S.; Sampaio, J. L.; Surendranath, V.; Duchoslav, E.; Ekroos, K.; Klemm, R. W.; et al. Global analysis of the yeast lipidome by quantitative

shotgun mass spectrometry. *Proc Natl Acad Sci U S A*. **2009**, *106*, 2136–2141.

45. Jewison, T.; Su, Y.; Disfany, F. M.; Liang, Y.; Knox, C.; Maciejewski, A.; et al. SMPDB 2.0: big improvements to the Small Molecule Pathway Database. *Nucleic Acids Res*. **2014**, *42*, D478–D484.

46. Dudek, J. Role of Cardiolipin in Mitochondrial Signaling Pathways. *Front Cell Dev Biol*. **2017**, *5*, 90.

SUPPORTING INFORMATION

Supplementary Table 1. Complete subject charactheristics.

BREAST CANCER CASES	AGE	MENOPAUSE	SMOKING	HYSTOLOGICAL TYPE	TNM	T	N	M	GRADE	MOLECULAR SUBTYPE	ESTROGEN RECEPTOR	PROEGESTERONE RECEPTOR	HER2	KI67	MEDICATION
BC1	34	no	yes	IDC + micropapillary component	IIA	T1c	N1	0	2	luminal A	60%	90%	neg	10%	no
BC2	42	no	no	IDC	IV	T4a	N1	1	3	luminal B HER2 +	95%	95%	3+	60%	no
BC3	51	yes	no	IDC	IIA	T2	N0	0	3	luminal B HER2-	0	10%	neg	90%	Losartan, meformin, indapamide
BC4	39	no	no	IDC	IIA	T2	N0	0	2	luminal A	80%	80%	neg	10%	no
BC5	42	no	yes	IDC	IA	T1c	N0	0	2	luminal A	90%	100%	neg	20%	no
BC6	48	no	yes	IDC	IIA	T2	N0	0	1	luminal A	100%	1%	neg	3-5%	losartan, Atenolol, levothyroxine
BC7	56	yes	no	IDC	IIA	T2	N0	0	3	HER2+	0	0	3+	30%	amlodipine, levothyroxine
BC8	68	yes	no	IDC	IA	T1	N0	0	2	TN	0	0	neg	40%	no
BC9	42	yes	no	IDC	IIIB	T4	N1	0	2	luminal B HER2 +	70%	80%	3+	30%	no
BC10	37	no	no	IDC	IIB	T2	N1	0	2	luminal A	90%	80%	neg	20%	no

BC11	56	yes	yes	IDC	IIIB	T4	N1	0	2	luminal A	95%	80%	neg	20%	no
BC12	35	no	no	IDC	IIA	T2	N0	0	2	luminal B HER2-	100%	90%	neg	50%	no
BC13	53	yes	no	IDC	IIIB	T4b	N1	0	2	luminal B HER2 +	100%	90%	3+	40%	no
BC14	47	no	no	IDC	IIB	T2	N1	0	2	HER2+	neg	neg	3+	10%	Amlodipine, hidroclorotiazide
BC15	71	yes	no	IDC	IV	T3	N0	1	2	luminal B HER2 +	90%	90%	3+	50%	no
BC16	55	no	no	IDC	IIA	T2	N0	0	3	HER2+	neg	neg	3+	50%	Captopril
BC17	51	yes	no	IDC	IV	T4	N3	1	1	luminal A	70%	10%	neg	20%	atenolol
BC18	37	no	no	IDC	IIA	T2	N0	0	3	TN	neg	neg	neg	80%	no
BC19	51	no	no	IDC	IIIB	T4	N0	0	2	luminal B HER2 +	70%	neg	3+	25%	Atenolol
BC20	53	yes	no	IDC	IIIC	T4d	N3	0	3	TN	neg	neg	neg	30%	no
BC21	39	no	no	IDC	IIA	T2	N0	0	2	luminal B HER2 +	95%	97%	3+	45%	no
BC22	42	no	no	IDC	IV	T4	N1	1	3	HER2+	neg	neg	3+	70%	no
BC23	44	no	no	IDC + squamous differentiation	IIB	T2	N0	0	3	HER2+	neg	neg	3+	50%	no

HEALTHY CONTROL SUBJECT	**AGE**	**MENOPAUSE**	**SMOKING**	**CHILDBEARING**	**MEDICATION**

S

HC1	28	no	no	0	Drospirenone, etinilestradiol, omeprazole
HC2	24	no	no	0	mebeverine
HC3	35	no	no	1	no
HC4	29	no	no	0	no
HC5	26	no	no	0	Drospirenone, etinilestradiol,
HC6	26	no	no	0	Drospirenone, etinilestradiol,
HC7	22	no	no	0	no
HC8	25	no	yes	0	no
HC9	44	no	no	0	no
HC10	31	no	no	2	no
HC11	50	yes	no	0	omeprazole
HC12	31	no	no	2	levothyroxine
HC13	47	no	no	0	levothyroxine
HC14	22	no	no	1	Drospirenone,

					etinilestradiol,
HC15	39	no	no	0	no
HC16	31	no	no	3	no
HC17	46	no	no	0	no
HC18	39	no	no	0	metformnin
HC19	37	no	no	0	no
HC20	38	no	no	3	metfottmin
HC21	73	yes	no	0	no
HC22	55	yes	no	4	no
HC23	39	no	no	2	no
HC24	37	no	no	0	atorvastatin
HC25	74	yes	no	2	losartan, levothyroxine, atorvastatin
HC26	44	no	no	3	no
HC27	41	no	no	1	no
HC28	55	yes	no	1	Anlodipina, telmisartan
HC29	65	yes	no	2	Metformin, sertraline, etinilestradiol

HC30	54	yes	no	2	Venlafaxine, etinilestradiol
HC31	48	yes	no	0	no
HC32	54	yes	no	1	Losartan, Hydrochlorothiazide, atorvastatin
HC33	54	yes	no	0	no
HC34	56	yes	no	3	no
HC35	51	yes	yes	2	no

BC: breast cancer, IDC: invasive ductal carcinoma, TNM: tumor node metastasis stage, T: tumor, N: node, M: metastasis, HC: healthy control.

Supplementary Table 2. Salivary metabolites previously reported with molecular weight and M/Z

metabolites	molecular weight	m/z (M+H)
2-Hydroxypentanoate	117.124	118.12
Dodecanoic acid	200	201.37
N-Acetylneuraminate	309	310.26
N-ε-Acetyllysine	188.224	189.23
1,3-Diaminopropane	74.124	75.13
2-hydroxy-4 methylvaleric acid	132.159	133.16
2-Hydroxy-4-methylpentanoate	131.151	132.15
2-Hydroxybutanedioate	132.071	133.07
2-Hydroxybutyric acid	104.104	105.10
2-Hydroxyvaleric acid	118.131	119.13
2-Ketobutyric acid	102.088	103.09
3-Hydroxybutyric acid	104.104	105.10
4- Methilbenzoate	135.142	136.14
4-Hydroxyphenylpyruvic acid	180.157	181.16
4-methoxyphenylacetic acid	166.173	167.17
4-Trimethylammoniobutanoic	146.207	147.21

acid		
5-Aminolevulinic acid	131.129	132.13
6,N6,N6-Trimethyl-L-lysine	188.267	189.27
7-Methylguanine	165.152	166.15
Adenosine	267.241	268,24
Alanyl-Alanine	160.171	161.17
alpha-Ketoisovaleric acid	116.115	117.12
Arachidic acid	312.530	313.53
arginine	174.201	175.20
Aspartic acid	133.102	133.10
beta-alanine	89.093	90.09
betaine	117.146	118.15
Butyric acid	88.105	89.11
c-aminobutyric acid	103.119	104.12
cadaverine	102.178	103.18
Caproic acid	116.158	117.16
choline	104.170	105.17
cis-Aconitate	174.108	175.11
Citric acid	192.123	193.12
citrulline	175.185	176.19
Cystine	240.3	241.30
Cytosine	111.102	112.10

D- alpha-aminobutyric acid	103.119	104.12
Desaminotyrosine	166.173	167.17
diaminopropane	74.124	75.13
Diglycine	132.119	133.12
Ethanolamine	61.083	62.08
Ethanolamine Phosphate	141.063	142.06
formic acid	46.025	47.03
glutamic acid	147.129	148.13
glutamine	146.144	147.14
Glutathione	307.323	308.32
glycine	75.066	76.07
glycolic acid	76.051	77.05
Glycyl-L-leucine	188.22	189.22
Guanine	151.126	152.13
Guanosine	283.240	284.24
heptanoic acid	130.184	131.18
histidine	155.154	156,15
homocysteine	135.185	136.19
Hydrocinnamic acid	150.174	151.17
Hydroxylysine	162.187	163.19
Hypotaurine	109.147	110.15
Hypoxanthine	136.111	137.11

Inosine	268.226	269.23
isoleucine	131.172	132.17
Isopropanolamine	75.110	76.11
L- alpha-aminobutyric acid	103.119	104.12
L-Alanine	89.093	90.09
L-carnitine	161.198	162.20
lactic acid	90.077	91.08
leucine	131.172	132.17
Leucinic acid	132.157	133.16
lysine	146.187	147.19
LysoPC (16:0)	495.630	496.63
LysoPC (18:1)	521.676	522.68
LysoPC (22:6)	567.694	568.69
LysoPE (18:2/0:0)	477.571	478.57
methionine	149.211	150.21
Myristic acid	228.370	229.37
N-Acetyl-L-phenylalanine	207.225	208.23
N-Acetylornithine	174.197	175.20
N.N-Dimethylglycin	103.121	104.12
N1 –acetylspermidine	187.282	188.28
N1-acetyl-spermine	244.376	245,38
N1-acetylputrescine	130.188	131.19

N6-Acetyl-L-lysine	188.224	189.22
N8-Acetylspermidine	187.282	188.28
Nicotinic acid	123.109	124.11
Octanoate	143.206	144.21
octanoic acid	256.424	257.42
ornithine	132.161	133.16
p-hydroxyphenylacetic acid	152.147	153.15
palmitic amid	284.477	285.48
phenylalanine	165.189	166.19
Phenyllactic acid	166.173	167.17
Phosphoserine	185.072	186.07
Pipecolic acid	129.157	130.16
piperidine	85.147	86.15
proline	115.130	116.13
putrescine	88.151	89.15
Pyrroline hydroxycarboxylic acid	129.114	130.11
Ribose 5-phosphate	230.110	231.11
saccharic acid derivative (N-Acetylneuraminic acid)	210.138	211.14
serine	105.092	106.09
spermidine	145.245	146.25

spermine	202.340	203.34
sphingolipid (phytosphingosine)	317.514	318.51
taurine	125.147	126.15
terephthalic acid	166.130	167.13
threonine	119.119	120.12
Trimethylamine	59.110	60.11
Tryptophan	204.225	205.23
tyrosine	181.188	182.19
urea	60.055	61.06
Ureidosuccinic acid	176.127	177.13
uric acid	168.110	169.11
valine	117.146	118.15

Supplementary Table 3. **List of medications used by subjects**

Medications	molecular mass	m/z DA	number of cases using	number of controls using
losartan	422.91 g/mol	423.91	2	2
metformin	129.16364 g/mol	130.16	1	2
indapamide	365.835 g/mol	366.83	1	0
Mebeverine	429.6 g/mol	430.6	0	1
Sertraline	306.229 g/mol	307.22	0	1
Clonazepam	315.715 g·mol	316.71	0	0
valsartan	435.519 g/mol	436.51	0	0
levothyroxine	776.874 $g \cdot mol^{-1}$	777.87	2	3
amlodipine	408.879 g/mol	409.87	2	1
Hydrochlorothiazide	297.74 g/mol	298.74	1	1
Captopril	217.29 g/mol	218.29	1	0
Atenolol	266.336 g/mo	267.33	3	0
drospirenone	366.493 g/mol	367.49	0	1
Omeprazole	345.42 g/mol	346.42	0	2
venlafaxine	277.402 g/mol	278.40	0	1
telmisartan	514.617 g/mol	515.61	0	1
ethinylestradiol	296.403 g/mol	297.40	0	3

Supplementary Table 4. Unidentified ion statistically overexpressed in patients with breast cancer

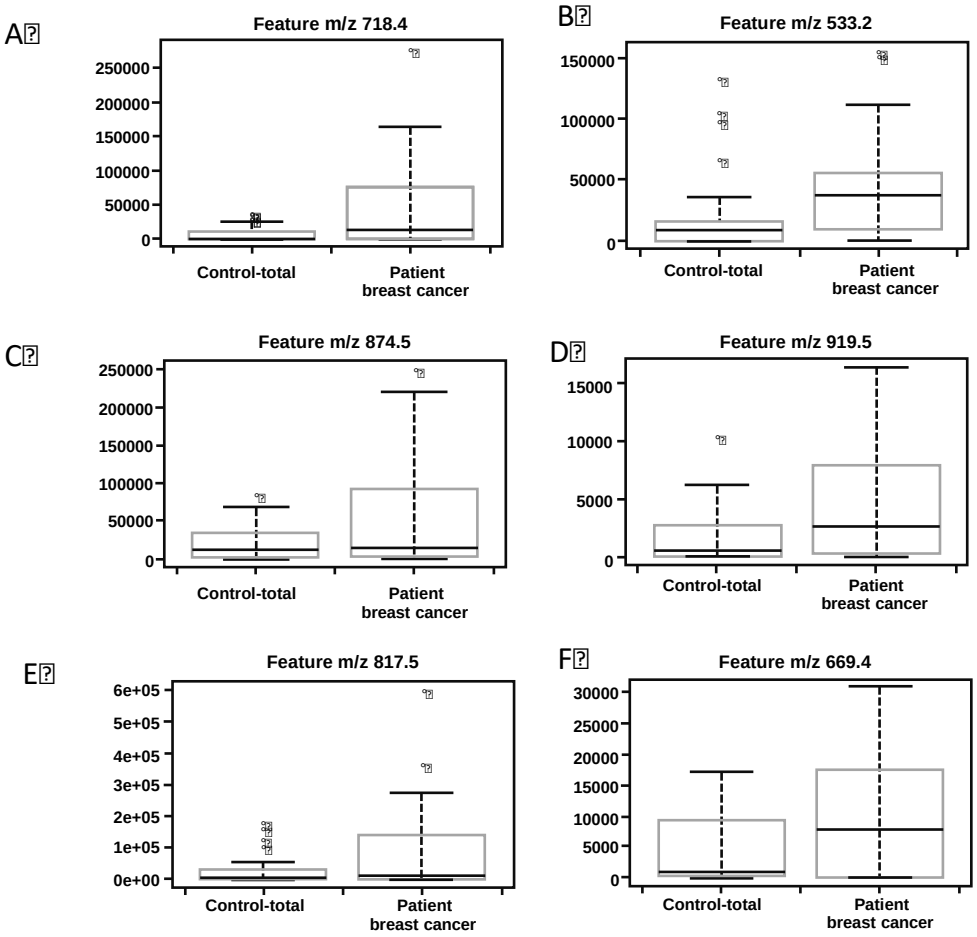
m/z	Retention time in HPLC	fold change (a)	p-value
356.69	3.7	5.8	0.02
413.22	2.6	2.0	0.03
456.72	2.6	3.3	0.002
457.73	2.5	2.8	0.02
467.20	3.0	2.3	0.02
467.71	2.8	3.3	0.01
475.70	2.6	3.4	0.002
534.23	2.5	1.9	0.04
594.82	3.0	2.2	0.04
602.32	2.7	3.5	0.04
602.96	3.1	2.5	0.01
614.32	2.6	2.4	0.01
614.82	2.8	3.0	0.04
661.34	3.0	2.7	0.04
749.4	3.0	2.3	0.02
818.47	2.4	3.2	0.04
875.49	2.5	2.8	0.04
1,015.04	3.1	2.4	0.03

.HPLC : high performance liquid chromatography; (a) Increasing fold change between the group of patients and of controls

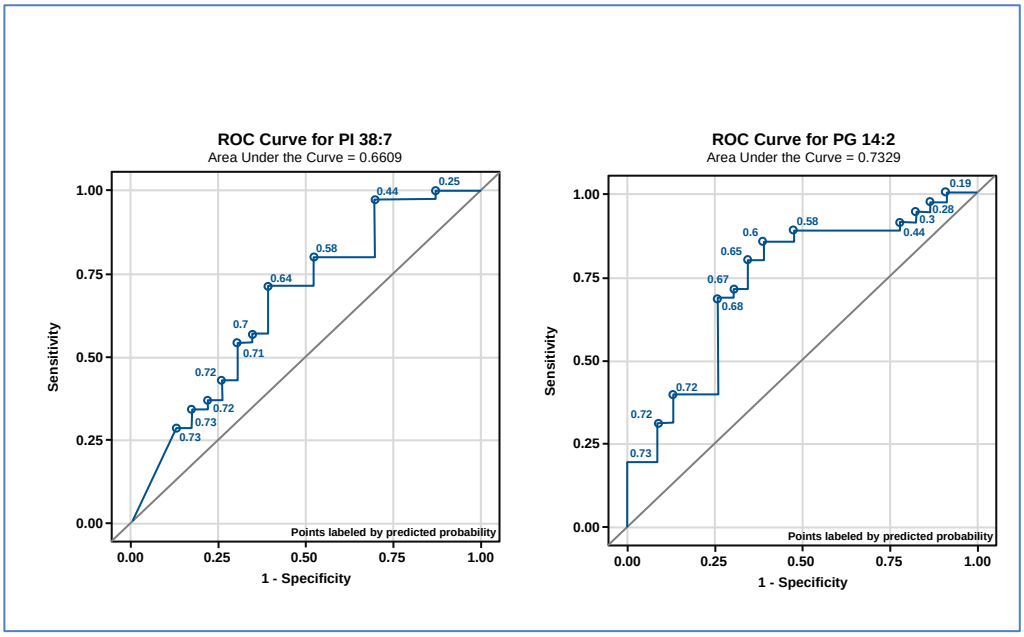
Supplementary Table 5. Treatment characteristics

Breast cancer cases	TNM	T	N	M	Grade	Molecular subtype	Treatment	response
BC3	IIA	T2	N0	0	3	luminal B HER2-	NACT: AC x 4 cycles- paclitaxel	CR
BC9	IIA	T2	N0	0	3	HER2+	NACT: Docetaxel + carboplatin + trastuzumabe	PR
BC10	IA	T1	N0	0	2	TN	NACT: AC x 4 cycles- carboplatin and paclitaxel	PR
BC13	IIIB	T4	N1	0	2	luminal A	NACT	PR
BC19	IIA	T2	N0	0	3	HER2+	NACT: AC x 4 cycles- paclitaxel+ pertuzumabe + trastuzumabe x 4 cycles	CR
BC20	IV	T4	N3	1	1	luminal A	Paliative tamoxifen	PR
BC21	IIA	T2	N0	0	3	TN	NACT: AC x 4 - paclitaxel	CR
BC24	IIA	T2	N0	0	2	luminal B HER2 +	NACT: AC x 4 cycles- Docetaxel + pertuzumabe + trastuzumabe	CR
BC25	IV	T4	N1	1	3	HER2+	Paliative treatment: Docetaxel + Pertuzumab+ trastuzumabx 6 cycles	PR
BC26	IIB	T2	N0	0	3	HER2+	NACT: AC x 4 cycles- paclitaxel+ pertuzumabe + trastuzumabe x 4 cycles	CR

BC: breast cancer, T: tumor, N: node, M: metastasis; TNM: Tumor Node Mestastasis system from the 7th edition of the American Joint Committee on Cancer guidelines.; NACT: neo-adjuvant chemotherapy; AC: doxorrubicin plus cyclophosphamide; CR: complete response; PR: partial response, TN: triple negative;



Supplementary Figure 1. Box and Whispers corresponding to the areas of the identified compounds in the two groups (control and patients). A m/z 718.4, B m/z 533.2, C: m/z 874.5, D: m/z 919.5, E m/z 817.5, F m/z 669.4



Supplementary Figure 2. ROC curves for PI 38:7 (AUC 0.6609, sensitivity 60.87% and specificity 71.43%) and PG 14:2 (AUC 0.7329, sensitivity 65.22% and specificity 77.14%)

3 DISCUSSION

A biomarker-based test has clinical utility if it demonstrates a favorable balance of benefits against harms. If clinical care options result in similar patient survival, biomarker tests may still have clinical utility if they direct care to options that result in improved quality of life (eg, less toxicity or inconvenience) or lower cost(89). A new biomarker test should contribute clinically with useful information beyond that already provided by standardly used clinical or pathologic indicators, unless the new test can provide equivalent information at a lower cost, less invasively, or with less inconvenience or risk(17). CEA (18), CA 15-3, and CA 27-29 may be used as adjunctive assessments to contribute to decisions regarding therapy for metastatic breast cancer(17). Data are insufficient to recommend use of CEA, CA 15-3, and CA 27-29 alone for monitoring response to treatment or diagnosis(90).

In the last decade, saliva emerged as a source of biochemical data, able to detect chronic diseases, as it may contain real-time information describing the overall physiological condition (91). The discovery of biomarkers in oral fluids allows the identification of molecules that can provide valuable information for the screening, detection and monitoring for solid tumors such as breast cancer (50, 77, 92). Despite promising emerging results, further research is needed in this area so that saliva can be effectively implemented in clinical practice as diagnostic fluid(93). There are few studies reporting the use of saliva for breast cancer detection(94). They identify and quantify cancer related proteins in saliva that were previously detected in serum and these first studies provide rational for the studies of saliva in cancer diagnosis(95).

The first article investigated the expression of CA15-3 protein in saliva and blood of breast cancer patients as a potential complementary strategy for diagnosis, prediction of disease progression and monitoring treatment efficacy. The gold standard for breast cancer screening and diagnosis, which consists of mammography and breast biopsy, respectively, entails significant limitations such as high cost and morbidity. Therefore, less inconvenient

procedures to ensure a safe technique should be developed. Moreover, it should be applied at all stages of breast cancer: screening, diagnosis, treatment and monitoring for metastasis prediction (123, 124). This unmet need to find an ideal biomarker for breast cancer may explain the large number of studies that have been addressing this theme since the year 1999.

CA15-3 is a large, often overexpressed and aberrantly glycosylated transmembrane protein in cancer that appears to play a role in cell adhesion (127). It is the most commonly used serum marker to detect breast cancer recurrence and to monitor the treatment of patients with advanced disease, since its expression increases in most cases (128). Circulating levels of CA15-3 have been shown to correlate with tumor size, reflecting the stage of the disease (129, 130). ECLIA is currently the most widely used technique for the processing of serum CA15-3, as it has high sensitivity and specificity as an analytical tool. Salivary evaluation of CA15-3 is more commonly performed in the context of clinical research by the ELISA technique.

We evaluated three methods ELISA, CLIA, and ECLIA for quantifying the concentration of CA15-3 in saliva and blood of patients with breast cancer. CLIA and ECLIA are used routinely in clinical exams for evaluation of serum tumor markers and sorology of viral infectious agents and for these reason those techniques were chosen for evaluate salivary CA15-3 (96). There are no previous reports of the use of CLIA and ECLIA in saliva. ECLIA and CLIA do not require long incubations and the addition of stopping reagents, as conventional colorimetric assays, such as ELISA, so they have superior low-end sensitivity, and a faster protocol. A method for detecting CA15-3 in saliva would be more comfortable and convenient for patients than current serum analyses that require venipuncture.

There was no significant difference between mean serum and salivary CA15-3 levels in patients comparing with healthy controls. However, the mean values for CA15-3 in serum were higher in breast cancer patients. There was a moderate correlation between salivary and serum CA15-3 levels in breast cancer patients while it was measured by ELISA ($r = 0.56$, $p = 0.0047$). The

results showed that ECLIA is not a good method to detect salivary CA15-3, although it is the golden standard for serum CA15-3.

Agha-Hosseini *et al*. evaluated the association between serum and salivary levels of CA15-3 and compared them between 26 women with breast cancer and 35 healthy women. Using the enzyme immunoassay assay on serum and salivary samples, the authors found that CA15-3 levels in blood and saliva were significantly higher in cancer patients, with a significant positive correlation between serum CA15-3 and saliva concentrations, suggesting the potential use of salivary CA15-3 in the initial detection of breast cancer (69). As with the study by Agha-Hosseini *et al*., Colomer *et al*. (95), Streckfus *et al*. (71, 101), Azeez *et al*. (132), Irfan *et al*. (133) and Atoum *et al*. (134) found similar results, with significant difference between serum and/ or salivary CA15-3 of breast cancer patients and healthy controls.

The reasons that could explain these different results could be: methodological differences, use of different kits and reagents, sample size, non-standard or non-reproducible assays, and misleading statistical analyzes. It is true that CA15-3 is expressed in saliva, however, we could not demonstrate in the present study that ECLIA, CLIA or ELISA techniques are able to discriminate cases from controls, although the mean values for CA15-3 in serum and saliva were higher in breast cancer patients.

The data found in the first article confirm the possibility of using saliva as a diagnostic fluid, due to the expression of CA15-3 in it. However, the fact that salivary CA15-3 values obtained by different methods do not correlate with serum CA15-3, except for the ELISA method in breast cancer patients, limits the use of this biomarker dosage in saliva for diagnostic and disease monitoring purposes. Future research should include more patients and different methods to determine which one is most appropriate.

The second article was a systematic review on salivary metabolites in cancer patients. The cancer researches through metabolomics could reveal new biomarkers that may be useful for future diagnosis, prognosis and therapy. The review aimed to evaluate salivary metabolites and their diagnostic value in cancer patients. Five electronic databases were searched.

Among 1,151 identified studies, 25 were included. 13 and 12 studies used targeted and untargeted metabolomics approaches, respectively to identify the value of salivary metabolites in diagnosing cancer. Among 140 salivary metabolites that demonstrated statistically significant differences between cancer patients and healthy controls, 46 were amino acids. Combinations of certain metabolites showed excellent sensitivity and specificity in the diagnosis of breast and oral cancers.

The process of oncogenesis is dependent on AA, the building blocks for protein synthesis, and a source of energy and metabolites. The essential AA may either be used for protein synthesis or be oxidized for the energy needs of tumors. In this review, 46 described metabolites were AA. All the branched-chain (essential) AAs were among those described in this review. Among the studies on breast cancer, the metabolites were only identified in Asian populations in 3 studies. In the study by Sugimoto *et al*., the ethnicity of the patients were not specified; therefore, these metabolites may not be generalized to all populations with BC(97).

The review showed that the majority of studies that used untargeted metabolomic approach did not make a further confirmation of the metabolites identified. There are few studies to evaluate metabolomics in untargeted approach for breast cancer and few studies included more than 50 subjects in each group. We believe that the study makes a significant contribution to the literature because although the metabolomics profiling approach is becoming increasingly popular, its value in the diagnosis of cancer has not been well investigated. In particular, salivary metabolomics provides a non-invasive approach, and the site of sample collection is easily accessible. It is therefore suitable for both, the early diagnosis and follow-up of patients with cancer.

In the third article, salivary metabolites of patients with breast cancer were determined using untargeted metabolomics. This prospective study evaluated saliva samples obtained from 23 breast cancer patients and 35 healthy controls, which were subjected to liquid chromatography and mass spectrometry. The results were processed using an online bioinformatics tool and some ions were identified using the METLIN database. We found that a

total of 31 ions were up-regulated in the breast cancer group, including 7 oligopeptides and 6 glycerophospholipids.

Peptides and AAs are derived from various sources, such as fragmented proteins from incomplete breakdown products of protein digestion or protein catabolism. Some peptides are known to have physiological or cell signaling effects, although most are simply short-lived intermediates on their way to specific AA degradation pathways following additional proteolysis, in addition, they may be the product of incomplete digestion or protein catabolism (98).

The results revealed no difference in salivary AAs between patients and controls, opposite to that sohwn by Cheng *et al*., Zhong *et al*., and Sugimoto *et al*.(52, 81, 99) All listed studies included at least 30 patients. Ours included 23, so this coud be one reason for divergent results. The methods of the three studies also were different from Ultra High Performance Liquid Chromatography-quadrupole time-of-flight mass spectrometry analysis used in our study. Finally, those three studies included Asiatic subjects, while ours included ocidental subjects. It is well described that metabolism can be different among different ethnic groups, so this could have impacted the analysis(100, 101). Since the available evidence is still scarce and the few studies in BC patients have small samples, further research in different populations is necessary.

Furthermore, we evaluated pre-treatment and post-treatment samples from a subset of 10 patients who experienced response to systemic treatment, which revealed that 4 ions (3 peptides and a glycerophospholipid) were up-regulated before treatment but not after treatment. Receiver operating characteristic curve analysis revealed that the salivary lipids provided good specificity and fair sensitivity for identifying breast cancer, although further large-scale studies are needed to validate our findings. Nevertheless, we believe that these results may be useful for guiding the implementation of metabolomics for identifying breast cancer and facilitating early intervention. This study makes a significant contribution since early identification and treatment of breast cancer is important to improve outcomes, although the

reference standard is breast biopsy and histotopathology studies. Thus, a simpler and less invasive tool would be useful. Saliva samples can be subjected to metabolomic analysis to identify changes in biomolecules, which may be useful in this setting.

One of the major questions regarding salivary biomarker is how large proteins enter the saliva proper because many proteins are too large to pass through the intercellular spaces of the acinar cells(102). Exosome-like microvesicles could be the underlying mechanism by which proteins enter saliva(103). Exosomes are membrane bound extra cellular vesicles that originate from late endosome, ranging in size from 30 to 150 nano meter that are released from several types of the cells and can be found circulating in almost all biological fluids(104). It is known that exosomes carry different molecular components of the cells from which they originate and can include proteins, lipids, microRNA and mRNA(105). The exosomes proteins can be either enclosed within the vesicles or present on surface membrane. Breast cancer exosomes interacts with cells of salivary gland, which in turn change the composition of salivary gland cell derived exosomes both proteomically and transcriptomically(106). Using an in vitro breast cancer model, Lau *et al*., demonstrated that breast cancer-derived exosome-like microvesicles are capable of interacting with salivary gland cells, altering the composition of their secreted exosome-like microvesicles and communicated and activated the transcriptional activity of the salivar glands(107).

Our long-term goal is to develop a noninvasive saliva-based tool for early detection of breast cancer. We envision a clinical context in which a salivary test can enable clinicians to detect breast cancer earlier (identifying patients who warrant closer follow-up and additional imaging) and reduce the number of unnecessary biopsies. Metabolomics studies may be useful to determination of this biomarker for breast cancer screnning and early detection.

4 CONCLUSION

From the three articles developed we can conclude that serum CA15-3 values were higher in breast cancer patients, but not for salivary CA15-3. ECLIA was not a good method to detect salivary CA15-3, although it is the golden standard for detecting serum CA15-3. In breast cancer patients, we observed a correlation between serum and salivary CA15-3 detected by ELISA. CA15-3 concentrations were highest in stage IV and luminal breast cancer subtypes. Further investigations are needed to confirm the capability of detection of salivary CA15-3 and its correlation to serum CA15-3.

Twenty five studies that utilized targeted and untargeted metabolomics approaches to identify the value of salivary metabolites in diagnosing cancer were included in a systematic review. Among 140 salivary metabolites that demonstrated statistically significant differences between cancer patients and healthy controls, 46 were AA. Proline, threonine, and histidine in combination demonstrated excellent sensitivity and specificity for diagnosing breast cancer. The highest DTA for diagnosing breast cancer was for MG (0:0/14:0/0:0).

LC/MS profiles of saliva of 23 breast cancer patients and 35 healthy controls by XCMS online were evaluated, in an untargeted approach. From the 31 up regulated ions, 13 metabolites had a possible identification in Metlin database: 7 peptides and 6 lipids (PG14:2, PA32:1, PS 28:0, PS 40:6, PI31:1 and PI38:7). Metabolite profiles before and after treatment in 10 patients, who had at least a partial response, showed that 3 peptides and PG 14:2 that were up-regulated before, returned to levels similar to healthy controls after treatment. Although the subgroup analysis of treatment is limited due to the few samples analysed, the search of a biomarker of response is extremely attractive.

In summary, we found that saliva is a fluid for researching breast cancer tumor markers and deserves further study. We do not expect fluid tumor marker search to replace standard screening and diagnostic methods with physical examination, mammography and biopsy. However, we can envision a possible scenario in which a new salivary test may increase the ability to detect breast cancer early when it is still curable with existing

treatments. Research on biomarkers in saliva may prove to be as useful as research on biomarkers in blood. Further studies to compare the metabolite profiles obtained concurrently from saliva, blood and cancer tissue is needed to provide rational evidence for the systemic metabolite links.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018(68):394-424.
2. INCA. Estimativa 2018: Insituto Nacional do Câncer; 2018 [Available from: http://www.inca.gov.br/estimativa/2018/index.asp.
3. INCA. Atlas de Mortalidade Brasil: Ministério da Saúde; 2019 [Available from: https://mortalidade.inca.gov.br/MortalidadeWeb/.
4. Byler S, Goldgar S, Heerboth S, Leary M, Housman G, Moulton K, et al. Genetic and epigenetic aspects of breast cancer progression and therapy. Anticancer Res. 2014;34(3):1071-7.
5. Howell A, Anderson AS, Clarke RB, Duffy SW, Evans DG, Garcia-Closas M, et al. Risk determination and prevention of breast cancer. Breast Cancer Research : BCR. 2014;16(5):446-.
6. Breastcancer.org. U.S. Breast Cancer Statistics 2019 [Available from: http://www.breastcancer.org.
7. Etzioni R, Urban N, Ramsey S, McIntosh M, Schwartz S, Reid B, et al. The case for early detection. Nature reviews Cancer. 2003;3(4):243-52.
8. Arellano M, Jiang J, Zhou X, Zhang L, Ye H, Wong DT, et al. Current advances in identification of cancer biomarkers in saliva. Frontiers in bioscience (Scholar edition). 2009;1:296-303.
9. Cardoso F, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rubio IT, et al. Early breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2019.
10. Berg WA, Gutierrez L, NessAiver MS, Carter WB, Bhargavan M, Lewis RS, et al. Diagnostic accuracy of mammography, clinical examination, US, and MR imaging in preoperative assessment of breast cancer. Radiology. 2004;233(3):830-49.
11. Drukteinis JS, Mooney BP, Flowers CI, Gatenby RA. Beyond mammography: new frontiers in breast cancer screening. Am J Med. 2013;126(6):472-9.
12. Zhang YJ, Wei L, Li J, Zheng YQ, Li XR. Status quo and development trend of breast biopsy technology. Gland surgery. 2013;2(1):15-24.
13. Hayes DF. Biomarker validation and testing. Mol Oncol. 2015;9(5):960-6.
14. Henry NL, Hayes DF. Cancer biomarkers. Mol Oncol. 2012;6(2):140-6.
15. Wagner PD, Srivastava S. New paradigms in translational science research in cancer biomarkers. Translational research : the journal of laboratory and clinical medicine. 2012;159(4):343-53.
16. Dasari S, Wudayagiri R, Valluru L. Cervical cancer: Biomarkers for diagnosis and treatment. Clin Chim Acta. 2015;445:7-11.
17. Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. Journal of Clinical Oncology. 2007;25(33):5287-312.
18. Cen B, Xiong Y, Song JH, Mahajan S, DuPont R, McEachern K, et al. The Pim-1 Protein Kinase Is an Important Regulator of MET Receptor Tyrosine Kinase Levels and Signaling. Molecular and Cellular Biology. 2014;34(13):2517-32.
19. Labianca R, Nordlinger B, Beretta GD, Mosconi S, Mandala M, Cervantes A, et al. Early colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2013;24 Suppl 6:vi64-72.

20. Schmoll HJ, Van Cutsem E, Stein A, Valentini V, Glimelius B, Haustermans K, et al. ESMO Consensus Guidelines for management of patients with colon and rectal cancer. a personalized approach to clinical decision making. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2012;23(10):2479-516.
21. Van Poznak C, Somerfield MR, Bast RC, Cristofanilli M, Goetz MP, Gonzalez-Angulo AM, et al. Use of Biomarkers to Guide Decisions on Systemic Therapy for Women With Metastatic Breast Cancer: American Society of Clinical Oncology Clinical Practice Guideline. Journal of Clinical Oncology. 2015;33(24):2695-704.
22. Amado FM, Ferreira RP, Vitorino R. One decade of salivary proteomics: current approaches and outstanding challenges. Clin Biochem. 2013;46(6):506-17.
23. Bonne NJ, Wong DT. Salivary biomarker development using genomic, proteomic and metabolomic approaches. Genome Medicine. 2012;4(10):82.
24. Cuevas-Cordoba B, Santiago-Garcia J. Saliva: a fluid of study for OMICS. OMICS. 2014;18(2):87-97.
25. Wang Q, Gao P, Wang X, Duan Y. The early diagnosis and monitoring of squamous cell carcinoma via saliva metabolomics. Scientific Reports. 2014;4:6802.
26. Zhang A, Sun H, Wang P, Wang X. Salivary proteomics in biomedical research. Clin Chim Acta. 2013;415:261-5.
27. Li Y, St John MA, Zhou X, Kim Y, Sinha U, Jordan RC, et al. Salivary transcriptome diagnostics for oral cancer detection. Clinical cancer research : an official journal of the American Association for Cancer Research. 2004;10(24):8442-50.
28. Hu S, Arellano M, Boontheung P, Wang J, Zhou H, Jiang J, et al. Salivary proteomics for oral cancer biomarker discovery. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(19):6246-52.
29. Bigler LR, Streckfus CF, Dubinsky WP. Salivary biomarkers for the detection of malignant tumors that are remote from the oral cavity. Clinics in laboratory medicine. 2009;29(1):71-85.
30. Gao K, Zhou H, Zhang L, Lee JW, Zhou Q, Hu S, et al. Systemic disease-induced salivary biomarker profiles in mouse models of melanoma and non-small cell lung cancer. PloS one. 2009;4(6):e5875.
31. Kaufman E, Lamster IB. The diagnostic applications of saliva--a review. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists. 2002;13(2):197-212.
32. Streckfus CF, Bigler LR. Saliva as a diagnostic fluid. Oral Dis. 2002;8(2):69-76.
33. Zhang L, Xiao H, Wong DT. Salivary biomarkers for clinical applications. Molecular diagnosis & therapy. 2009;13(4):245-59.
34. Mandel ID. The diagnostic uses of saliva. J Oral Pathol Med. 1990;19(3):119-25.
35. Taylor-Papadimitriou J, Burchell J, Miles DW, Dalziel M. MUC1 and cancer. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 1999;1455(2):301-13.
36. Kufe DW. Mucins in cancer: function, prognosis and therapy. Nature reviews Cancer. 2009;9(12):874-85.
37. Bafna S, Kaur S, Batra SK. Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells. Oncogene. 2010;29(20):2893-904.
38. Duffy MJ. Biochemical markers in breast cancer: which ones are clinically useful? Clin Biochem. 2001;34(5):347-52.
39. Hayes DF, Sekine H, Ohno T, Abe M, Keefe K, Kufe DW. Use of a murine monoclonal antibody for detection of circulating plasma DF3 antigen levels in breast cancer patients. The Journal of Clinical Investigation. 1985;75(5):1671-8.
40. Klee GG, Schreiber WE. MUC1 gene-derived glycoprotein assays for monitoring breast cancer (CA 15-3, CA 27.29, BR): are they measuring the same antigen? Arch Pathol Lab Med. 2004;128(10):1131-5.
41. Duffy MJ, Evoy D, McDermott EW. CA 15-3: uses and limitation as a biomarker for breast cancer. Clin Chim Acta. 2010;411(23-24):1869-74.

42. Emens LA, Davidson NE. The follow-up of breast cancer. Seminars in oncology. 2003;30(3):338-48.
43. Kallioniemi OP, Oksa H, Aaran RK, Hietanen T, Lehtinen M, Koivula T. Serum CA 15-3 assay in the diagnosis and follow-up of breast cancer. British Journal of Cancer. 1988;58(2):213-5.
44. Colomer R, Ruibal A, Genolla J, Rubio D, Del Campo JM, Bodi R, et al. Circulating CA 15-3 levels in the postsurgical follow-up of breast cancer patients and in non-malignant diseases. Breast cancer research and treatment. 1989;13(2):123-33.
45. Touitou Y, Bogdan A. Tumor markers in non-malignant diseases. European journal of cancer & clinical oncology. 1988;24(7):1083-91.
46. Colomer R, Ruibal A, GenollÃ J, Salvador L. Circulating CA 15-3 antigen levels in non-mammary malignancies. British Journal of Cancer. 1989;59(2):283-6.
47. Streckfus C, Bigler L, Tucci M, Thigpen JT. A preliminary study of CA15-3, c-erbB-2, epidermal growth factor receptor, cathepsin-D, and p53 in saliva among women with breast carcinoma. Cancer Invest. 2000;18(2):101-9.
48. Bafna S, Kaur S, Batra SK. Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells. Oncogene. 2010;29(20):2893-904.
49. Al-Tarawneh SK, Border MB, Dibble CF, Bencharit S. Defining salivary biomarkers using mass spectrometry-based proteomics: a systematic review. Omics. 2011;15(6):353-61.
50. Zhang L, Xiao H, Karlan S, Zhou H, Gross J, Elashoff D, et al. Discovery and preclinical validation of salivary transcriptomic and proteomic biomarkers for the non-invasive detection of breast cancer. PloS one. 2010;5(12):e15573.
51. Patel S, Ahmed S. Emerging field of metabolomics: big promise for cancer biomarker identification and drug discovery. J Pharm Biomed Anal. 2015;107:63-74.
52. Sugimoto M, Wong DT, Hirayama A, Soga T, Tomita M. Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. Metabolomics. 2010;6(1):78-95.
53. DeBerardinis RJ, Chandel NS. Fundamentals of cancer metabolism. Science advances. 2016;2(5):e1600200-e.
54. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
55. Hanahan D, Weinberg Robert A. Hallmarks of Cancer: The Next Generation. Cell. 2011;144(5):646-74.
56. Dang CV. Links between metabolism and cancer. Genes & development. 2012;26(9):877-90.
57. Jang M, Kim SS, Lee J. Cancer cell metabolism: implications for therapeutic targets. Experimental &Amp; Molecular Medicine. 2013;45:e45.
58. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. Nature medicine. 2004;10(8):789-99.
59. Kroemer G, Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell. 2008;13(6):472-82.
60. Ždralević M, Marchiq I, de Padua MMC, Parks SK, Pouysségur J. Metabolic Plasiticy in Cancers-Distinct Role of Glycolytic Enzymes GPI, LDHs or Membrane Transporters MCTs. Frontiers in Oncology. 2017;7:313-.
61. Gandhi N, Das GM. Metabolic Reprogramming in Breast Cancer and Its Therapeutic Implications. Cells. 2019;8(2):89.
62. Corbet C, Feron O. Cancer cell metabolism and mitochondria: Nutrient plasticity for TCA cycle fueling. Biochimica et biophysica acta Reviews on cancer. 2017;1868(1):7-15.
63. Ray U, Roy SS. Aberrant lipid metabolism in cancer cells - the role of oncolipid-activated signaling. Febs j. 2018;285(3):432-43.
64. Choi Y-K, Park K-G. Targeting Glutamine Metabolism for Cancer Treatment. Biomol Ther (Seoul). 2018;26(1):19-28.

65. Cannino G, Ciscato F, Masgras I, Sánchez-Martín C, Rasola A. Metabolic Plasticity of Tumor Cell Mitochondria. Frontiers in Oncology. 2018;8:333-.
66. Kuo C-Y, Ann DK. When fats commit crimes: fatty acid metabolism, cancer stemness and therapeutic resistance. Cancer Commun (Lond). 2018;38(1):47-.
67. Anderson NM, Mucka P, Kern JG, Feng H. The emerging role and targetability of the TCA cycle in cancer metabolism. Protein Cell. 2018;9(2):216-37.
68. Holeček M. Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. Nutr Metab (Lond). 2018;15:33-.
69. Lohavanichbutr P, Zhang Y, Wang P, Gu H, Nagana Gowda GA, Djukovic D, et al. Salivary metabolite profiling distinguishes patients with oral cavity squamous cell carcinoma from normal controls. PloS one. 2018;13(9):e0204249.
70. Emwas AH, Roy R, McKay RT, Tenori L, Saccenti E, Gowda GAN, et al. NMR Spectroscopy for Metabolomics Research. Metabolites. 2019;9(7).
71. Gentric G, Mieulet V, Mechta-Grigoriou F. Heterogeneity in Cancer Metabolism: New Concepts in an Old Field. Antioxidants & redox signaling. 2017;26(9):462-85.
72. Barnes S, Benton HP, Casazza K, Cooper SJ, Cui X, Du X, et al. Training in metabolomics research. I. Designing the experiment, collecting and extracting samples and generating metabolomics data. J Mass Spectrom. 2016;51(7):461-75.
73. Patti GJ, Yanes O, Siuzdak G. Innovation: Metabolomics: the apogee of the omics trilogy. Nat Rev Mol Cell Biol. 2012;13(4):263-9.
74. Zhou B, Xiao JF, Ressom HW. Prioritization of putative metabolite identifications in LC-MS/MS experiments using a computational pipeline. Proteomics. 2013;13(2):248-60.
75. Roberts LD, Souza AL, Gerszten RE, Clish CB. Targeted metabolomics. Curr Protoc Mol Biol. 2012;Chapter 30:Unit30.2-.2.24.
76. Dettmer K, Aronov PA, Hammock BD. Mass spectrometry-based metabolomics. Mass spectrometry reviews. 2007;26(1):51-78.
77. Guerra EN, Acevedo AC, Leite AF, Gozal D, Chardin H, De Luca Canto G. Diagnostic capability of salivary biomarkers in the assessment of head and neck cancer: A systematic review and meta-analysis. Oral oncology. 2015;51(9):805-18.
78. Guerra EN, Rego DF, Elias ST, Coletta RD, Mezzomo LA, Gozal D, et al. Diagnostic accuracy of serum biomarkers for head and neck cancer: A systematic review and meta-analysis. Crit Rev Oncol Hematol. 2016;101:93-118.
79. Wang Q, Gao P, Wang X, Duan Y. Investigation and identification of potential biomarkers in human saliva for the early diagnosis of oral squamous cell carcinoma. Clin Chim Acta. 2014;427:79-85.
80. Wang Q, Gao P, Cheng F, Wang X, Duan Y. Measurement of salivary metabolite biomarkers for early monitoring of oral cancer with ultra performance liquid chromatography–mass spectrometry. Talanta. 2014;119:299-305.
81. Zhong L, Cheng F, Lu X, Duan Y, Wang X. Untargeted saliva metabonomics study of breast cancer based on ultra performance liquid chromatography coupled to mass spectrometry with HILIC and RPLC separations. Talanta. 2016;158:351-60.
82. Nunes LA, Mussavira S, Bindhu OS. Clinical and diagnostic utility of saliva as a non-invasive diagnostic fluid: a systematic review. Biochem Med (Zagreb). 2015;25(2):177-92.
83. Patti GJ, Yanes O, Siuzdak G. Innovation: Metabolomics: the apogee of the omics trilogy. Nat Rev Mol Cell Biol. 2012;13(4):263-9.
84. Cox JT. 1 Epidemiology of cervical intraepithelial neoplasia: the role of human papillomavirus. Bailliere's Clinical Obstetrics and Gynaecology. 1995;9(1):1-37.
85. Jessie K, Jayapalan JJ, Ong KC, Abdul Rahim ZH, Zain RM, Wong KT, et al. Aberrant proteins in the saliva of patients with oral squamous cell carcinoma. Electrophoresis. 2013;34(17):2495-502.
86. Malhotra R, Urs AB, Chakravarti A, Kumar S, Gupta VK, Mahajan B. Correlation of Cyfra 21-1 levels in saliva and serum with CK19 mRNA expression in oral squamous cell

carcinoma. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2016;37(7):9263-71.
87. de Vos FYFL, Nuver J, Willemse PHB, van der Zee AGJ, Messerschmidt J, Burgerhof JGM, et al. Long-term survivors of ovarian malignancies after cisplatin-based chemotherapy: cardiovascular risk factors and signs of vascular damage. European Journal of Cancer. 2004;40(5):696-700.
88. Gacche RN, Meshram RJ. Angiogenic factors as potential drug target: Efficacy and limitations of anti-angiogenic therapy. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer. 2014;1846(1):161-79.
89. Harris LN, Ismaila N, McShane LM, Andre F, Collyar DE, Gonzalez-Angulo AM, et al. Use of Biomarkers to Guide Decisions on Adjuvant Systemic Therapy for Women With Early-Stage Invasive Breast Cancer: American Society of Clinical Oncology Clinical Practice Guideline. Journal of Clinical Oncology. 2016;34(10):1134-50.
90. Van Poznak C, Somerfield MR, Bast RC, Cristofanilli M, Goetz MP, Gonzalez-Angulo AM, et al. Use of Biomarkers to Guide Decisions on Systemic Therapy for Women With Metastatic Breast Cancer: American Society of Clinical Oncology Clinical Practice Guideline. Journal of Clinical Oncology. 2015;33(24):2695-704.
91. Schafer CA, Schafer JJ, Yakob M, Lima P, Camargo P, Wong DT. Saliva diagnostics: utilizing oral fluids to determine health status. Monographs in oral science. 2014;24:88-98.
92. Porto-Mascarenhas EC, Assad DX, Chardin H, Gozal D, De Luca Canto G, Acevedo AC, et al. Salivary biomarkers in the diagnosis of breast cancer: A review. Crit Rev Oncol Hematol. 2017;110:62-73.
93. Miller CS, Foley JD, Bailey AL, Campell CL, Humphries RL, Christodoulides N, et al. Current developments in salivary diagnostics. Biomarkers in medicine. 2010;4(1):171-89.
94. Abrao Nemeir I, Saab J, Hleihel W, Errachid A, Jafferzic-Renault N, Zine N. The Advent of Salivary Breast Cancer Biomarker Detection Using Affinity Sensors. Sensors (Basel). 2019;19(10):2373.
95. Liu X, Yu H, Qiao Y, Yang J, Shu J, Zhang J, et al. Salivary Glycopatterns as Potential Biomarkers for Screening of Early-Stage Breast Cancer. EBioMedicine. 2018;28:70-9.
96. Victer TN, Dos Santos CS, Bao SN, Sampaio TL. Deceased tissue donor serology and molecular testing for HIV, hepatitis B and hepatitis C viruses: a lack of cadaveric validated tests. Cell and tissue banking. 2016;17(4):543-53.
97. Sugimoto M, Wong D, Soga T, Tomita M. Salivary metabolomics for oral cancer detection. Oral Cancer: Causes, Diagnosis and Treatment. 2011:271-8.
98. Wishart DS, Feunang YD, Marcu A, Guo AC, Liang K, Vazquez-Fresno R, et al. HMDB 4.0: the human metabolome database for 2018. Nucleic acids research. 2018;46(D1):D608-d17.
99. Cheng F, Wang Z, Huang Y, Duan Y, Wang X. Investigation of salivary free amino acid profile for early diagnosis of breast cancer with ultra performance liquid chromatography-mass spectrometry. Clin Chim Acta. 2015;447:23-31.
100. de Boer JO, van Es AJ, Voorrips LE, Blokstra F, Vogt JE. Energy metabolism and requirements in different ethnic groups. European journal of clinical nutrition. 1988;42(12):983-97.
101. O'Donnell PH, Dolan ME. Cancer pharmacoethnicity: ethnic differences in susceptibility to the effects of chemotherapy. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15(15):4806-14.
102. Lee Y-H, Wong DT. Saliva: an emerging biofluid for early detection of diseases. Am J Dent. 2009;22(4):241-8.
103. Nonaka T, Wong DTW. Saliva-Exosomics in Cancer: Molecular Characterization of Cancer-Derived Exosomes in Saliva. The Enzymes. 2017;42:125-51.
104. Sumrin A, Moazzam S, Khan AA, Ramzan I, Batool Z, Kaleem S, et al. Exosomes as Biomarker of Cancer. Brazilian Archives of Biology and Technology. 2018;61.

105. Roy S, Lin H-Y, Chou C-Y, Huang C-H, Small J, Sadik N, et al. Navigating the Landscape of Tumor Extracellular Vesicle Heterogeneity. International journal of molecular sciences. 2019;20(6):1349.
106. Lucchetti D, Fattorossi A, Sgambato A. Extracellular Vesicles in Oncology: Progress and Pitfalls in the Methods of Isolation and Analysis. Biotechnology Journal. 2019;14(1):1700716.
107. Lau CS, Wong DTW. Breast cancer exosome-like microvesicles and salivary gland cells interplay alters salivary gland cell-derived exosome-like microvesicles in vitro. PloS one. 2012;7(3):e33037-e.

APPENDIX -APPROVAL OF THE ETHICS COMMITTEE

UNB - FACULDADE DE CIÊNCIAS DA SAÚDE

Continuação do Parecer: 1.767.139

Metodologia:

- "A coleta da saliva será realizada após o diagnóstico histopatológico de neoplasia de mama, cancer do colo do útero e câncer de cabeça e pescoço, além dos controles saudáveis. Os participantes serão instruídos a não comer, beber, fumar ou realizar procedimentos de higiene oral por pelo menos 1 hora antes da coleta de saliva. Para a coleta, um cotonete de algodão (Salivette®, Sarstedt AG & Co, Nümbrecht, Oberbergischer Kreis, Alemanha) será inserido na boca dos participantes por um período de 5 minutos. A amostra de saliva será centrifugada por 10 minutos a 1500 rpm a 4ºC. Posteriormente, a saliva será transferida para um tubo e diluída (1:1) numa solução salina tamponada com fosfato (PBS) (0,4mM de NaCl e 10mM de NaPO4) contendo inibidores de protease (0,01 mM de EDTA; 0,01 mg/mL de aprotinina A) e 0,05% de Tween-20. A solução será homogeneizada e congelada a -80ºC até o momento das análises.";
- "As reações de ELISA (Ensaio imunoenzimático) serão realizadas utilizando os Kits TGF-B1 (DY240, DuoSet, R&D Systems, Minneapolis, MN, USA) e IL-10 (DY2117BE, DuoSet, R&D Systems, Minneapolis, MN, USA) de acordo com as especificações do fabricante...";
- "PCR array: Utilizando placas customizadas (SABioscience), serão avaliados a expressão de genes relacionados ao diagnóstico do câncer de mama e do câncer de cabeça e pescoço, além de 3 genes de referência para normalização das reações. A reação será realizada com o sistema ABI StepOnePlus (SABioscience), utilizando o SYBR® Green qPCR Mastermix (SABioscience), seguindo as recomendações do fabricante.";
- Critério de Inclusão: "Participantes portadores de câncer de cabeça e pescoço, câncer de mama e câncer do colo do útero que coletem a saliva antes do início do tratamento sistêmico ou radioterápico e que coletem a saliva após o fim do tratamento sistêmico ou radioterápico.";
- Critério de Exclusão: "Participantes portadores de câncer de mama, colo do útero e cabeça e pescoço que farão apenas tratamento cirúrgico; • Participantes portadores de câncer de mama, colo do útero e cabeça e pescoço e participantes do grupo controle que apresentarem sinais de morbidade, problemas de saúde como doença autoimune, HIV, alterações da função renal, insuficiência cardíaca congestiva, infecção ativa e hepatite.";

Hipótese: "A expressão dos biomarcadores na saliva de paciente com câncer de mama, câncer do colo do útero e câncer de cabeça e pescoço pode ter relevância diagnóstica e sofrer influência do tratamento.".

Endereço: Faculdade de Ciências da Saúde - Campus Darcy Ribeiro
Bairro: Asa Norte **CEP:** 70.910-900
UF: DF **Município:** BRASILIA
Telefone: (61)3107-1947 **E-mail:** cepfsunb@gmail.com

Objetivo da Pesquisa:

Objetivo Primário: "Desenvolver ferramentas eficientes e inovadoras utilizando a saliva como método diagnóstico para o Câncer.".

Objetivo Secundário: "- Normalizar as amostras: um estudo preliminar será realizado para analisar a variabilidade das amostras de acordo com o método de amostragem e conservação salivar; - Quantificar os marcadores salivares de câncer de mama, do colo do útero e de cabeça e pescoço;- Comparar o desempenho diagnóstico dos testes de referência (ELISA, qPCR) para a detecção de marcadores de proteínas salivares. - Comparar a expressão dos biomarcadores na saliva dos pacientes portadores de câncer de mama, câncer do colo do útero e câncer de cabeça e pescoço antes e após o tratamento.".

Avaliação dos Riscos e Benefícios:

Riscos: "Os riscos de participar da pesquisa são muito pequenos. Pode haver desconforto local ou ânsia de vômito decorrentes do contato do algodão com a boca e para minimizar os riscos, o senhor(a) será orientado(a) a não ingerir alimentos ou líquidos uma hora antes da coleta da saliva. Caso ocorra qualquer tipo de reação, a equipe médica do hospital sempre dará assistência, para que esses riscos sejam evitados ou diminuídos. Se você aceitar participar da pesquisa, estará contribuindo para a avaliação de novas formas de diagnóstico do câncer. Outro risco do estudo é o de gerar angústia aos participantes quanto ao resultado da análise de sua saliva. Para reduzir esse risco em qualquer momento, o (a) participante poderá ter acesso aos resultados e eles poderão ser publicados em eventos e revistas científicas sempre mantendo o sigilo da sua participação. Essas estratégias reduzem a possibilidade de danos às diversas dimensões da pessoa humana (moral, psicológica, social ou espiritual).".

Benefícios: "A pesquisa não oferece benefícios diretos e imediatos aos participantes, mas como benefícios futuros, espera-se que a pesquisa permita avaliar uma nova técnica diagnóstica não invasiva para os tumores malignos de mama, colo do útero e cabeça e pescoço, tendo em vista que o diagnóstico dessas doenças atualmente é feito por procedimentos invasivos de biópsia com envio do material para análise histopatológica.".

Comentários e Considerações sobre a Pesquisa:

Os pesquisadores responderam adequadamente à solicitação deste CEP, conforme elencado no

último ponto do parecer - número 1.698.527, emitido em 26 de agosto de 2016 (Considerações finais a critério do CEP), enviando carta resposta, com as devidas informações e apontamentos necessários para a análise do projeto.
Não foram observados óbices éticos.

Considerações sobre os Termos de apresentação obrigatória:
Documentos que compõem o processo:

1. Informações Básicas do Projeto: "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_720272.pdf", postado em 30/06/2016;
2. Folha de rosto: "folhaderosto.pdf", postado em 30/06/2016. Documento devidamente redigido e assinado;
3. Projeto Detalhado / Brochura Investigador: "projetocep.docx", postado em 28/06/2016. Documento contendo o projeto detalhado do estudo;
4. Outros: "cettroaceite.pdf", postado em 22/06/2016. Documento devidamente redigido, assinado por uma das instituições coparticipantes;
5. Outros: "cacon.pdf", postado em 21/06/2016. Documento devidamente redigido, assinado por uma das instituições coparticipantes;
6. Outros: "encaminhamentoaocep.pdf", postado em 20/06/2016. Documento devidamente redigido e assinado.
7. Declaração de pesquisadores: "compromissopesquisador.pdf", postado em 16/06/2016. Documento devidamente redigido e assinado;
8. Outros: "encaminhamentoaoCEP.docx", postado em 16/06/2016. Documento devidamente redigido, não assinado;
9. Outros: "CACON.docx", postado em 16/06/2016. Documento devidamente redigido, não assinado;
10. Outros: "cettro.docx", postado em 16/06/2016. Documento devidamente redigido, não assinado;
11. Declaração de pesquisador: "compromisso.docx"; postado em 16/06/2016. Documento devidamente redigido, não assinado;
12. Outros: "siriolibanes.doc", postado em 16/06/2016. Documento devidamente redigido, não assinado;
13. Outros: "termosirio.pdf", postado em 16/06/2016. Documento devidamente redigido e

assinado;

14. Outros: "adrianacastelo.pdf", postado em 16/06/2016. Currículo Lates de pesquisador do projeto;

15. Outros: "tatianastrava.pdf", postado em 31/06/2016. Currículo Lates de pesquisador do projeto;

16. Outros: "perola.pdf", postado em 31/06/2016. Currículo Lates de pesquisador do projeto;

17. Outros: "eliete.pdf", postado em 31/06/2016. Currículo Lates de pesquisador do projeto;

18. Outros: "gabriel.pdf", postado em 31/06/2016. Currículo Lates de pesquisador do projeto;

19. Outros: "yanna.pdf", postado em 31/06/2016. Currículo Lates de pesquisador do projeto;

20. Outros: "anacarolina.pdf", postado em 31/06/2016. Currículo Lates de pesquisador do projeto;

21. Outros: "elisa.pdf", postado em 31/06/2016. Currículo Lates de pesquisador do projeto;

22. Outros: "danielaxavierassad.pdf", postado em 31/06/2016. Currículo Lates de pesquisador do projeto;

23. TCLE / Termos de Assentimento / Justificativa de Ausência: "TCLE.docx", postado em 31/06/2016. Considerações sobre o documento serão realizadas em seção posterior;

24. Orçamento: "Orcamento.docx", postado em 30/05/2016. Documento contendo planilha orçamentária detalhada;

25. Cronograma: "CRONOGRAMA.docx", postado em 30/05/2016. Documento contendo cronograma detalhado.

Documentos incluídos ao processo após parecer nº 1.645.876, emitido em 23/07/2016:

1. Informações Básicas do Projeto: "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_720272.pdf", postado em 02/08/2016;

2. TCLE / Termos de Assentimento / Justificativa de Ausência: "TCLE.docx", postado em 02/06/2016. Considerações sobre o documento serão realizadas em seção posterior;

3. Outros: "cartarespostaaocep.pdf", postada em 02/08/2016. Documento contendo carta resposta ao parecer consubstanciado emitido em 23/07/2016;

4. Projeto Detalhado / Brochura Investigador: "projetocep.docx", postado em 02/06/2016. Documento contendo o projeto detalhado do estudo.

Documentos incluídos ao processo após parecer consubstanciado nº 1.698.527, emitido em 26 de agosto de 2016:

1. Informações Básicas do Projeto: "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_720272.pdf", postado em 19/09/2016;
2. Outros: "cartarespostaaocep.pdf", postada em 19/09/2016. Documento contendo carta resposta ao parecer consubstanciado emitido em 26/08/2016;
3. Outros: "cartarespostaaoapospendenciasCEP.docx", postada em 19/09/2016. Documento contendo carta resposta ao parecer consubstanciado emitido em 26/08/2016;
4. Projeto Detalhado / Brochura Investigador: "projetocep.docx", postado em 19/09/2016. Documento contendo o projeto detalhado do estudo;
5. TCLE / Termos de Assentimento / Justificativa de Ausência: "TCLE.docx", postado em 19/09/2016. Considerações sobre o documento serão realizadas em seção posterior.

Recomendações:
Não se aplica

Conclusões ou Pendências e Lista de Inadequações:
1) Os autores definiram como critérios de inclusão: "Participantes portadores de câncer de cabeça e pescoço, câncer de mama e câncer do colo do útero que coletem a saliva antes do início do tratamento sistêmico ou radioterápico e que coletem a saliva após o fim do tratamento sistêmico ou radioterápico; • Estar de acordo e assinar o Termo de Consentimento Livre e Esclarecido.". A assinatura do Termo de consentimento não deve ser considerada critério de inclusão. Solicita-se revisão desta seção;
Análise após parecer nº 1.645.876, emitido em 23/07/2016, e carta reposta dos pesquisadores:
PENDÊNCIA ATENDIDA. O critério de inclusão foi adequado.

2) Os autores definiram como riscos do projeto: "Os riscos aos participantes do projeto são mínimos, pois será feito apenas coleta da saliva. Nesse sentido, o método de coleta pode levar a desconforto local ou náusea decorrentes do contato do cotonete com a mucosa bucal.". Considerando-se que, segundo a Resolução CNS 466/2012, item V, "Toda pesquisa com seres humanos envolve risco em tipos e gradações variados". E ainda, em seu item II.22, que risco da pesquisa é a "possibilidade de danos à dimensão física, psíquica, moral, intelectual, social, cultural ou espiritual do ser humano, em qualquer pesquisa e dela decorrente". Solicita-se apresentar

análise de riscos e as formas de minimização dos mesmos.Tal revisão deve ser realizada nos projetos detalhado e na plataforma Brasil e no TCLE.
Análise após parecer nº 1.645.876, emitido em 23/07/2016, e carta reposta dos pesquisadores:
PENDÊNCIA NÃO ATENDIDA. Os autores realizaram modificações, no entanto, estas ainda contemplam somente riscos físicos da participação do indivíduo no estudo. Os pesquisadores são encorajados a discutir também as dimensões "psiquica, moral, intelectual, social, cultural ou espiritual do ser humano", e as formas de minimização destes riscos, conforme normas vigentes do país, elencadas no parecer anterior.

Análise após parecer nº 1.698.527, emitido em 26 de agosto de 2016, e carta reposta dos pesquisadores:
PENDÊNCIA ATENDIDA: os autores informam sobre outros "riscos", que não somente os físicos. As modificações foram satisfatórias, permitindo a aprovação do projeto.

3) Solicita-se esclarecer a forma de financiamento da Fundação Universidade de Brasília. Se for por meio de Edital, favor incluir o número do mesmo.

Análise após parecer nº 1.645.876, emitido em 23/07/2016, e carta reposta dos pesquisadores:
PENDÊNCIA ATENDIDA. Informação solicitada foi adicionada ao projeto.

4) Quanto ao TCLE:
a. Solicita-se adequar a linguagem ao perfil dos participantes: linguagem clara, sem termos técnicos;

Análise após parecer nº 1.645.876, emitido em 23/07/2016, e carta reposta dos pesquisadores:
PENDÊNCIA ATENDIDA. A linguagem se encontra mais adequada ao público estudado.

b. Solicita-se acrescentar tempo da pesquisa e de cada fase;

Análise após parecer nº 1.645.876, emitido em 23/07/2016, e carta reposta dos pesquisadores:
PENDÊNCIA ATENDIDA. A informação foi acrescentada.

c. Solicita-se revisão dos riscos e formas de minimizá-los;

Análise após parecer nº 1.645.876, emitido em 23/07/2016, e carta reposta dos pesquisadores: PENDÊNCIA PARCIALMENTE ATENDIDA. A análise de riscos ainda se encontra insatisfatória (favor levar em consideração item 2 das Conclusões deste parecer).

Análise após parecer nº 1.698.527, emitido em 26 de agosto de 2016, e carta reposta dos pesquisadores: PENDÊNCIA ATENDIDA: os autores informam sobre outros "riscos", que não somente os físicos. As modificações foram satisfatórias, permitindo a aprovação do projeto.

d. Solicita-se acrescentar informação sobre indenização de acordo com o item "II.7 - indenização – cobertura material para reparação a dano, causado pela pesquisa ao participante da pesquisa", sendo "II.6 – dano associado ou decorrente da pesquisa - agravo imediato ou posterior, direto ou indireto, ao indivíduo ou a coletividade, decorrente da pesquisa", conforme Resolução CNS 466/2012.";

Análise após parecer nº 1.645.876, emitido em 23/07/2016, e carta reposta dos pesquisadores: PENDÊNCIA ATENDIDA. A informação foi acrescentada.

e. Solicita-se correção de erros de digitação.

Análise após parecer nº 1.645.876, emitido em 23/07/2016, e carta reposta dos pesquisadores: PENDÊNCIA ATENDIDA. Documento foi revisto e adequado. Conclusão da análise das respostas às pendências do parecer consubstanciado nº 1.645.876, emitido em 23/07/2016.

Conclusão: Todas as pendências foram atendidas. Não há óbices éticos para a realização deste projeto. Protocolo de pesquisa está em conformidade com a Resolução CNS 466/2012 e Complementares.

Considerações Finais a critério do CEP:
A realização das atividades do projeto está condicionada à aprovação pelos CEPs responsáveis das instituições co-participantes.

De acordo com a Resolução 466/12 CNS, itens X.1.- 3.b. e XI.2.d, os pesquisadores responsáveis deverão apresentar relatórios parcial semestral e final do projeto de pesquisa, contados a partir da data de aprovação do protocolo de pesquisa.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_720272.pdf	19/09/2016 19:01:53		Aceito
Outros	cartarespostacep.pdf	19/09/2016 19:00:39	Daniele Xavier Assad	Aceito
Outros	cartarespostaaoapospendenciasCEP.docx	19/09/2016 19:00:15	Daniele Xavier Assad	Aceito
Projeto Detalhado / Brochura Investigador	projetocep.docx	19/09/2016 18:41:12	Daniele Xavier Assad	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.docx	19/09/2016 18:40:53	Daniele Xavier Assad	Aceito
Outros	cartarespostaaocep.pdf	02/08/2016 20:53:44	Daniele Xavier Assad	Aceito
Folha de Rosto	folhaderosto.pdf	30/06/2016 11:58:54	Daniele Xavier Assad	Aceito
Outros	cettroaceite.pdf	22/06/2016 15:40:21	Daniele Xavier Assad	Aceito
Outros	cacon.pdf	21/06/2016 15:13:59	Daniele Xavier Assad	Aceito
Outros	encaminhamentoaocep.pdf	20/06/2016 15:34:02	Daniele Xavier Assad	Aceito
Declaração de Pesquisadores	comprimissopesquisador.pdf	16/06/2016 16:25:20	Daniele Xavier Assad	Aceito
Outros	encaminhamentoaoCEP.docx	16/06/2016 15:14:49	Daniele Xavier Assad	Aceito
Outros	CACON.docx	16/06/2016 15:12:49	Daniele Xavier Assad	Aceito
Outros	cettro.docx	16/06/2016 15:11:37	Daniele Xavier Assad	Aceito
Declaração de Pesquisadores	compromisso.docx	16/06/2016 15:10:24	Daniele Xavier Assad	Aceito
Outros	siriolibanes.doc	16/06/2016 15:07:28	Daniele Xavier Assad	Aceito

UNB - FACULDADE DE CIÊNCIAS DA SAÚDE

Continuação do Parecer: 1.767.139

Outros	termosirio.pdf	16/06/2016 15:04:37	Daniele Xavier Assad	Aceito
Outros	adrianacastelo.pdf	16/06/2016 11:53:56	Daniele Xavier Assad	Aceito
Outros	tatianastrava.pdf	31/05/2016 22:06:23	Daniele Xavier Assad	Aceito
Outros	perola.pdf	31/05/2016 22:05:47	Daniele Xavier Assad	Aceito
Outros	eliete.pdf	31/05/2016 22:05:23	Daniele Xavier Assad	Aceito
Outros	gabriel.pdf	31/05/2016 22:03:28	Daniele Xavier Assad	Aceito
Outros	yanna.pdf	31/05/2016 22:02:41	Daniele Xavier Assad	Aceito
Outros	anacarolina.pdf	31/05/2016 22:01:20	Daniele Xavier Assad	Aceito
Outros	elisa.pdf	31/05/2016 22:00:19	Daniele Xavier Assad	Aceito
Outros	danielexavierassad.pdf	31/05/2016 21:59:24	Daniele Xavier Assad	Aceito
Orçamento	Orcamento.docx	30/05/2016 20:56:16	Daniele Xavier Assad	Aceito
Cronograma	CRONOGRAMA.docx	30/05/2016 20:54:27	Daniele Xavier Assad	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BRASILIA, 08 de Outubro de 2016

Assinado por:
Keila Elizabeth Fontana
(Coordenador)

Endereço: Faculdade de Ciências da Saúde - Campus Darcy Ribeiro
Bairro: Asa Norte **CEP:** 70.910-900
UF: DF **Município:** BRASILIA
Telefone: (61)3107-1947 **E-mail:** cepfsunb@gmail.com