Effect of Radiation on Oral Cancer patients by 1H NMR based Metabolomic

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Abstract

Oral microflora is well-orchestrated and acts as an in-order defense mechanism for any infection related to oral disease. It has an encouraging outcome in terms of early recovery biomarkers. The saliva of oral cancer patients' subjects and controls have been evaluated by 1 H NMR spectroscopy in search of possible early metabolic differences that could be obtained to see the eradication of disease that favors the symbiotic condition. The study employed 1 H NMR spectroscopy on 102 human saliva samples (including case and controls) and their spectral data were further subjected to multivariate and quantitative analysis.

The 1H NMR study of oral cancer samples shows clear demarcation and profound metabolic differences when compared with the controls. Several metabolites such as lactate, ethanol, succinate and glutamate were found to be of higher significance in oral cancer patients in contrast to controls. Significant metabolites could be considered as early repair markers for oral cancer patients as they are being restored to achieve symbiosis. The study, therefore, concluded the early recovery process of the diseased subjects with the restoration of possible metabolomic profiles similar to the controls.

Keywords: Radiation, Oral Cancer Patients, Metabolomic.

Introduction

Oral cancers count with roughly 40,000 new cases and 8,000 deaths recorded in the United States in 2013[21]. Cancer is the leading cause of death in economically developed countries[17]. Cancer is a becoming a major health problem also in India, with approximately 1 million cases occurring each year. Head and neck cancer are among the commonest cancers diagnosed in Indian subcontinent. Cancer accounts for 8% of the deaths in India[4].

The increasing number of oral cancer cases is a cause of major concern as it is associated with high morbidity and mortality. The primary reason for this unusually high incidence of HNC in India is the indiscriminate use of chewing tobacco in its various forms. In tobacco users, the oral cavity bears the brunt of the carcinogen and nearly 80,000-100,000 oral cancers are diagnosed every year in the country[4].

Material and Methods

Subjects and sample collection: The study involves a collection of 102 human saliva samples, in which oral cancer subjects (n = 77) and controls (n = 25) are mentioned in table 1. Saliva was collected in a sterile container (containing 2.0 mg of Sodium Azide) for 10 min of time duration in early morning hours 10AM to 12 PM. Patient consents were taken well before the study and were fully briefed about the investigation purpose. Samples were snap frozen in liquid nitrogen immediately after expectoration and later kept at -80°C till further processing of samples. TSP (Trimethylsilyl propionate) was used as an internal standard for all saliva samples.

According to the Gardner et al, the use of TSP as an internal standard has a significant impact on the condition where the buffer is not being used with saliva samples. Notably, TSP is known to bind protein which results in line broadening of signals obtained, but this happens more in plasma rather than in saliva, which has very low protein content. The saliva samples were collected in three phases during RT, after 2-3 weeks of RT and after completion of RT or post radiation.

Ethical approval: This study was ethically approved by ethical committee of Dr. RMLIMS, LKO.

Sample preparation and acquisition: Sample preparation and acquisition were performed in a similar way as mentioned in earlier study[15]. Samples were taken out of − 80 °C and thawed at room temperature and were further subjected to centrifugation at 5000×g for a duration of 30 min. The supernatant of the amount of 300 µl was isolated from each sample and was mixed with an equal amount (300 µl) of sodium phosphate buffer in 5 mm standard NMR tube (Wilmad Glass USA) and sample homogeneity was maintained by vortexing it for 30 s. Samples were subjected to NMR experiments by using spectrometer equipped with a 5 mm triple resonance inverse (TCI)1H/13C/15N cryoprobe with a Z-shielded gradient and standard vertical bore, operating at a proton frequency of 800.21 MHz (18.8 T) of Bruker Bio-spin Advance III 800 MHz NMR (Bruker, GmBH).

Statistical analysis (data reduction and pattern recognition): Phase baseline corrected and calibrated CPMR NMR Spectra were employed in multivariate analysis. The spectral region was reduced with a specific region in chemical shift after removing water region from 5.15 to 4.00. Binning and other removal of discrete regions were exactly performed as mentioned in previous research[5].

The resulting data matrices having normalized integral values were exported into Microsoft office Excel 2007. The resulting data matrix containing normalized spectral bins (or features) was then subjected to multivariate and univariate statistical analysis using different modules of MetaboAnalyst (4.0, a freely available, user-friendly, web-based analytical platform for metabolomics data analysis from the University of Alberta, Canada: www.metaboanalyst.ca)[1,8]. The analysis was performed following procedure as described previously.

Results

A representative class of 1H NMR-spectra obtained from oral cancer cases (Pre, during and post radiation) and control are presented in fig. 1.

In the given comparative spectra, we can easily identify the differences which were further illustrated by their metabolomic signatures representing some inter individual variability with respect to lactate within the cases group and control.

The spectra of the cases and controls group metabolites, show 38 metabolites obtained from pre, during and post-radiation and control. Those metabolites are shown in table 1.

Pathways impact and VIP score analysis of cases vs control: VIP score and pathway impact analysis were further executed for data enrichment using MetaboAnalyst (version 4.0) software along with SPSS (SPSS Inc., Version 21) software for independent student t test between the oral cancer case and control. All significant metabolites analyzed through VIP score of cases and controls are portrayed in Fig. 2 listing glutamate followed by proline, galactose, fucose, tyrosine, serine, phenylalanine, threonine, glycine, isoleucine, leucine and alanine found to be high in cases and control subjects. The pathway analysis (Fig. 2) reflected alanine, aspartate and glutamate metabolism which impacted the most followed by glycolysis/gluconeogenesis, butanoate metabolism, arginine and proline metabolism, citrate metabolism and pyruvate metabolism.

Significant fifteen metabolites from the saliva of oral cancer patients of pre-radiation therapy, during radiation and post-radiation therapy were studied. We obtained the maximum significant f-value (40.16) and the p-value (0.001) was for phenylalanine, whereas the lowest f-value (2.714) and p-value (0.049) were for glycine

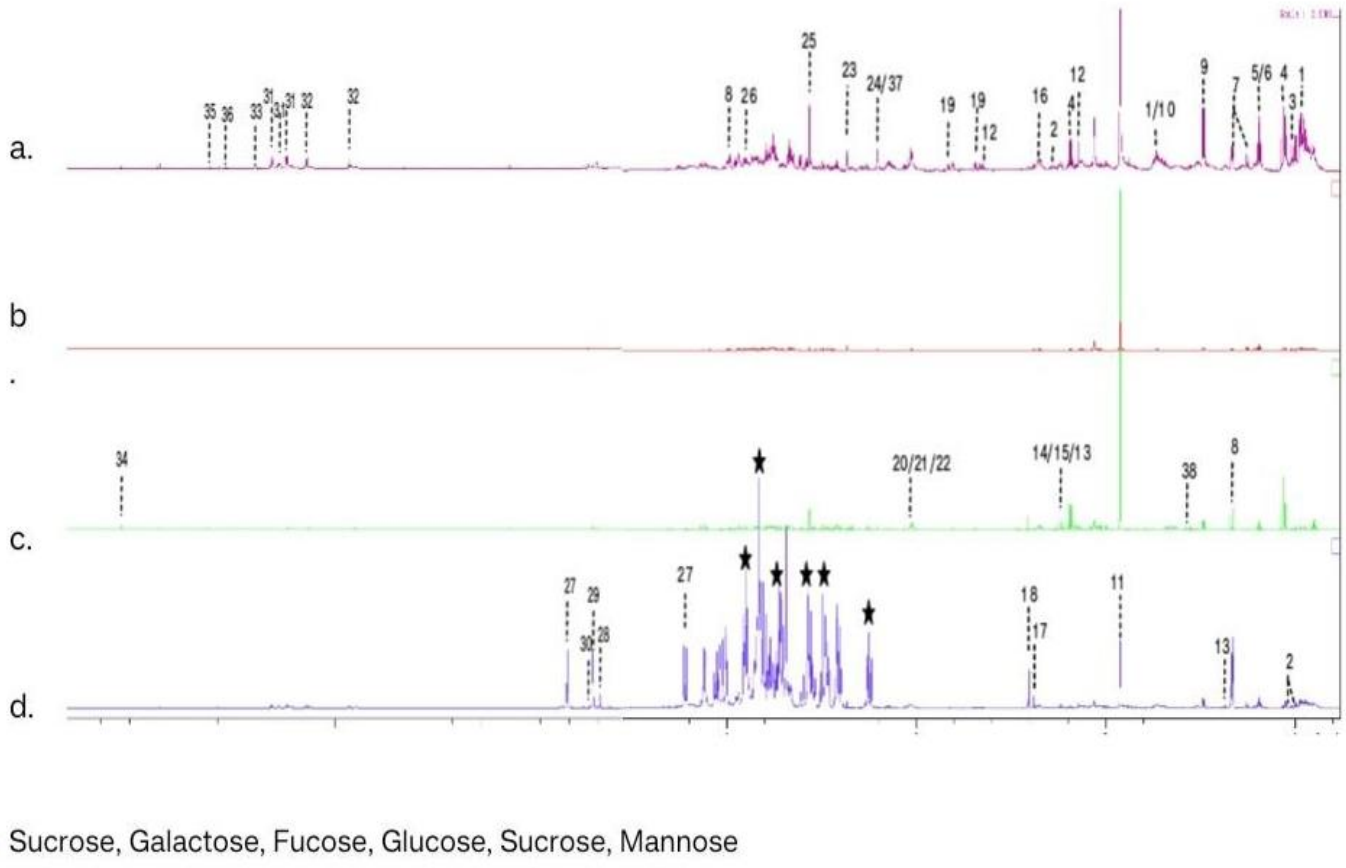

Fig. 1: Typical 800 MHz comparative 1H-NMR spectra of saliva samples of a. Post Radiotherapy b. During Radiotherapy c. Pre-Radiotherapy d. Healthy control were recorded at 300k

Table 1
List of Metabolites present in the Saliva samples of Cases (pre-RT, during RT, post RT) and Controls as per NMR analysis.

Labelling	Metabolites
1	Leucine
2	Valine
3	Isoleucine
4	Propionate
5/6	Ethanol/ Isoproenol
6	Ethanol
7	Fucose
8	Lactate
9	Alanine
1/10	Lysine/ Leucine
11	Acetate
12	Methionine
14/15/13	5-Aminopantonate/Acetone/Acetoin
14	5-Aminopantonate
15	Acetone
16	Glutamate
17	Pyruvate
18	Succinate
19	Aspartate
20	Creatine phosphate
21	Creatinine
22	Creatine
23	Methanol
24	Choline
25	Glycine
26	Serine
27	Sucrose
28	Mannose
29	Glucose
30	Galactose
31	Phenylalanine
32	Tyrosine
33	Uracil
34	Formate
35	2-Dioxyuredine
36	Thymidine
37	Phosphocholine
38	Butyrate

Table 2
Metabolites in saliva samples that significantly differ between control subjects and patients with oral cancer (before, during and after radiation therapy).

S.N.	Metabolites	f-value	p-value	Coefficient value
1.	Phenylalanine	40.164	0.001	0.01
2.	Isoleucine	38.966	0.001	0.01
3.	Acetate	30.66	0.001	2.2
4.	Tyrosine	23.116	0.001	0.02
5.	Alanine	20.795	0.001	0.02
6.	Threonine	19	0.001	0.02
7.	Glutamate	16.409	0.001	0.19
8.	Proline	12.612	0.001	0.08
9.	Galactose	9.1659	0.001	0.03
10.	Propionate	7.9994	0.001	0.02
11.	Serine	7.1322	0.002	0.01
12.	Butyrate	6.21	0.007	0.01
13.	Methanol	3.7217	0.014	0.02
14.	Fucose	3.6237	0.016	0.02
15.	Glycine	2.7143	0.049	0.01

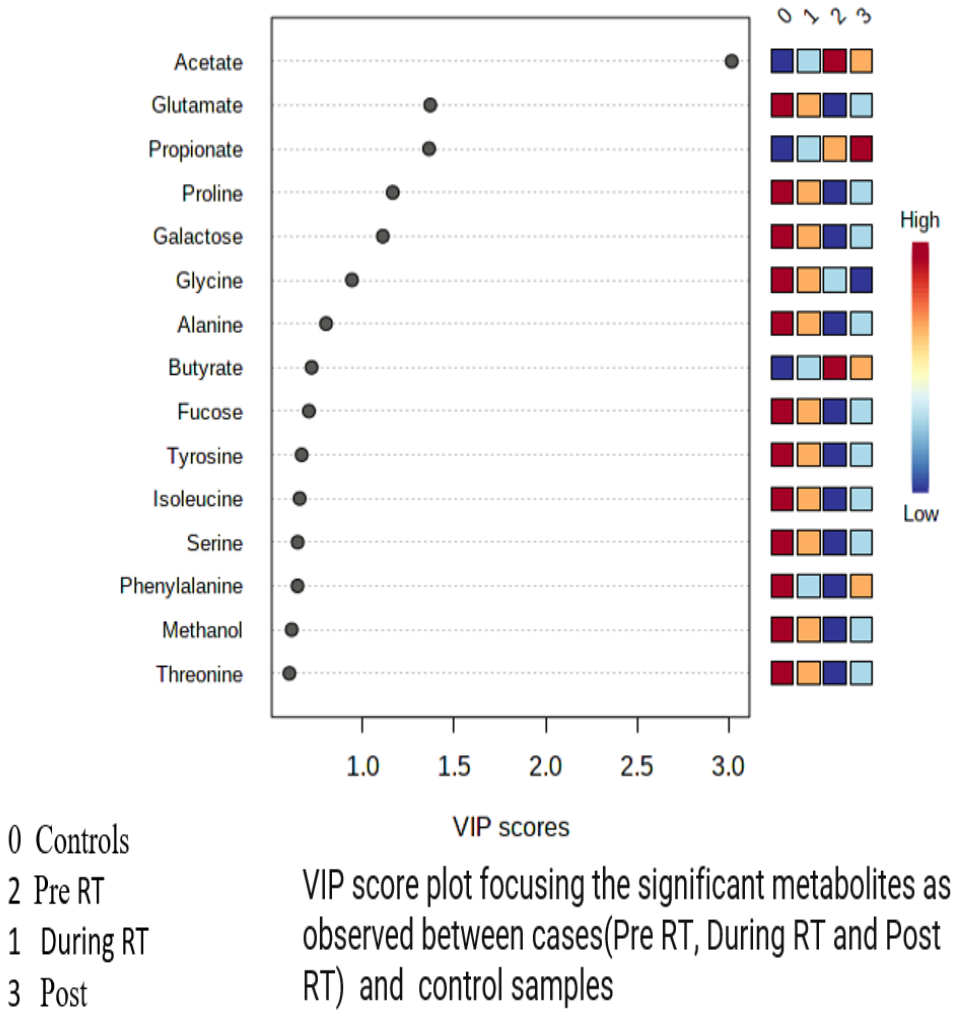

Figure 2: Distribution of significant metabolites in saliva samples from control subjects and patients with oral cancer: before, during and after radiation therapy.

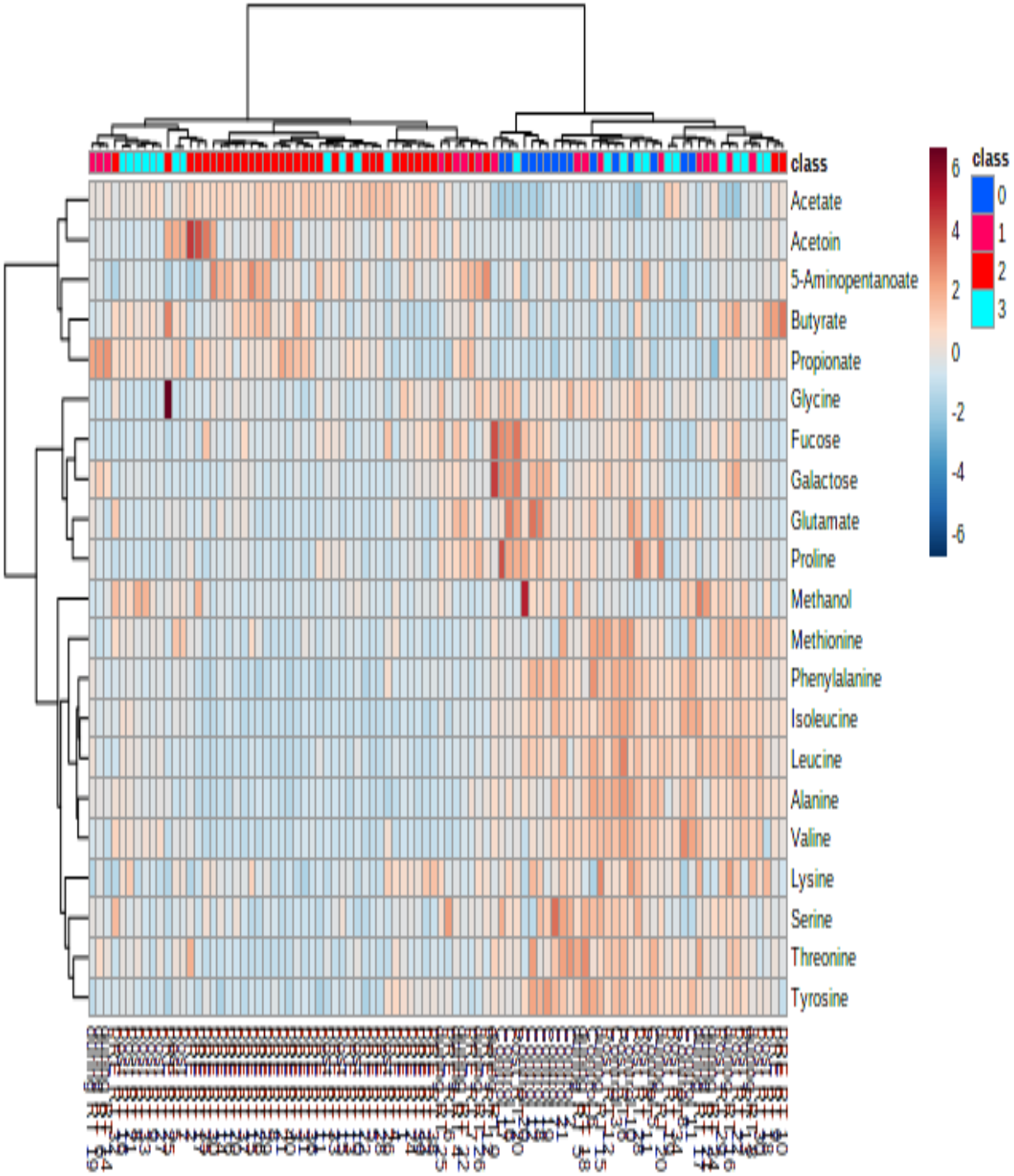

Figure 3: Heatmap analysis of 21 significant metabolites differentially abundant of oral cancer patients during pre-radiotherapy mid-radiotherapy and post radiotherapy and control group.

Discussion

Most of the population in developing or developed countries ignores or casually takes of oral cancer which may arise due to the lack of pain and sometimes bleeding gums until it has reached a chronic stage. This results in dragging the severe clinically detectable diseased subject to the oral cancer. For detection of oral cancer, saliva testing, a non-invasive alternative to serum testing, is rapidly advancing in recent years. Additionally, it is inexpensive and easy to use. The collection of saliva can reduce the discomfort for patients, particularly if repeated sampling is necessary. In our experiment, the metabolites obtained included glutamate, proline, galactose, fucose, tyrosine, serine, phenylalanine, threonine, glycine, isoleucine, leucine, alanine, acetate butyrate, propionate etc. in accordance with the human metabolomic database.

The 1H Nuclear Magnetic Resonance spectra from spittle in cases [pre-RT, mid-RT (3-4 weeks of RT and Post RT)] and controls (Total n=102) were analyzed for characterizing the

metabolites according to literature[7,11,19]. The assignment of metabolites was reinstated according to the biological resonance bank (BMRB, www.bmrb.wisc.edu)[6], Human Metabolome Information Base (HMDB, www. hmdb.ca)[11] and Chenomx version eight (Chenomx Iraqi National Congress).

Wei et al[20] distributed the substance signatures of oral malignant neoplastic disease and leucoplakia, indicating the superiority in levels of essential amino acid, carboxylic acid and essential amino acid in oral leukoplakia and oral cancers as compared to its controls, thereby providing activity USA with valuable facts about the metabolites expressed in oral malignancies[11].

As a result, the metabolomic process and glutaminolysis are regarded as the essential modalities and routes involved in cancer growth[16]. Furthermore, aspartate, glutamate, lactate, pyruvate, citrate and aminoalkanoic acid are formed when the amino acid is lysed[12]. This was confirmed in our study since the salt content in body cavity cancer patients was much higher than in healthy controls.

Overall significant metabolites (Cases/Controls) from the current investigation are based on statistical calculations using the ANOVA method. Glutamic acid and methionine have been identified as biomarkers for oral cancer[3]. Branched chain amino Acids (BCAA)-valine, leucine and isoleucine have been discovered to raise and play an important role not just in oral cancer but also in other forms of cancer. Because cancer cells require energy, BCAA metabolism is vital in energy generation in oral cancer cells[9]. Alanine is an amino acid that is commonly produced from BCAA and pyruvate. It is vital in proliferative cell metabolism and is a biomarker of severe hypoxia[13,14,18.]

Several amino acids: glycine and proline are defined as collagen motifs that influence tumor growth and cancer cell migration, invasion, proliferation, survival and metastasis. As a result, an increase in amino acid content may equate to an increase in collagen synthesis[2,20]. Changes in the oral and/or general atmosphere due to cancer medical care may result in the growth of several microorganisms and plant species in oral fissures, resulting in clinical bacterial/fungal infection. It is envisaged that even with oral/topical antimicrobials in patients with oropharyngeal cancer, the effect on irradiation of oral mucositis would be insignificant.

Conclusion

This data can aid in preventing infections in such patients including irradiation mucositis during or post-radiotherapy. This present study explored differences in salivary metabolites among patients before, during and post-radiotherapy, as compared to control and found that some metabolites were higher in cases and metabolite was lower in controls. Early detection of oral cancer by salivary metabolite screening is associated with a high expectation of preventing deformity, relapse and mortality.

References

1. Aimetti M., Cacciatore S., Graziano A. and Tenori L., Metabonomic analysis of saliva reveals generalized chronic periodontitis signature, *Metabolomics*, **8(3)**, 465-74 **(2012)**

2. American Society of Clinical Oncology **(2005-2011)**

3. Ben-Yoseph O., Badar-Goffer R.S., Morris P.G. and Bachelard H.S., Glycerol 3-phosphate and lactate as indicators of the cerebral cytoplasmic redox state in severe and mild hypoxia respectively: a 13C-and 31P-nmr study, *Biochemical Journal*, **291(3)**, 915-9 **(1993)**

4. Coelho K.R., Challenges of the oral cancer burden in India, *J Cancer Epidemiol*., doi: 10.1155/2012/701932 **(2012)**

5. Carpenter G.H., The secretion, components and properties of saliva, *Annual Review of Food Science and Technology*, **4**, 267-76 **(2013)**

6. Cruzat V., Macedo Rogero M., Noel Keane K., Curi R. and Newsholme P., Glutamine: metabolism and immune function, supplementation and clinical translation, *Nutrients*, **10(11)**, 1564 **(2018)**

7. Dame Z.T., Aziat F., Mandal R., Krishnamurthy R., Bouatra S., Borzouie S., Guo A.C., Sajed T., Deng L., Lin H. and Liu P., The human saliva metabolome, *Metabolomics*, **11(6)**, 1864-83 **(2015)**

8. Epstein J.B., Vickars L., Spinelli J. and Reece D., Efficacy of chlorhexidine and nystatin rinses in prevention of oral complications in leukemia and bone marrow transplantation, *Oral Surgery, Oral Medicine, Oral Pathology*, **73(6)**, 682-9 **(1992)**

9. Khanna R., Kumar K. and Roy R., A case study of primary malignancy of buccal mucosa using proton HR-MAS NMR spectroscopy on tissue specimens, *Journal of Oral Biology and Craniofacial Research*, **8(1)**, 68-72 **(2018)**

10. Markley J.L., Brüschweiler R., Edison A.S., Eghbalnia H.R., Powers R., Raftery D. and Wishart D.S., The future of NMR-based metabolomics, *Curr Opin Biotechnol*., **43**, 34-40, doi: 10.1016/j.copbio.2016.08.001 **(2017)**

11. Ogawa T., Washio J., Takahashi T., Echigo S. and Takahashi N., Glucose and glutamine metabolism in oral squamous cell carcinoma: insight from a quantitative metabolomic approach, *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*. **118(2)**, 218-25 **(2014)**

12. Ohshima M., Sugahara K., Kasahara K. and Katakura A., Metabolomic analysis of the saliva of Japanese patients with oral squamous cell carcinoma, *Oncology Reports*, **37(5)**, 2727-34 **(2017)**

13. Qi Y. and Xu R., Roles of PLODs in collagen synthesis and cancer progression, *Frontiers in Cell and Developmental Biology*, **6**, 66 **(2018)**

14. Rai V., Bose S., Saha S., Kumar V. and Chakraborty C., Delineating metabolic dysfunction in cellular metabolism of oral submucous fibrosis using 1H nuclear magnetic resonance spectroscopy, *Archives of Oral Biology*, **97**, 102-8 **(2019)**

15. Singh M.P., Saxena M., Saimbi C.S., Siddiqui M.H. and Roy R., Post-periodontal surgery propounds early repair salivary biomarkers by 1H NMR based metabolomics, *Metabolomics*, **15(11)**, 1-1 **(2019)**

16. Sridharan G. and Patankar S.R., Salivary metabolomics and oral carcinogenesis, *Journal of Tumor*, **4(4)**, 450-5 **(2016)**

17. Thun M.J., DeLancey J.O., Center M.M., Jemal A. and Ward E.M., The global burden of cancer: priorities for prevention, *Carcinogenesis*, **1**, 100-10, doi: 10.1093/carcin/bgp263 **(2010)**

18. Vittore L., Westbrook R.L. and Tennant D.A., New aspects of amino acid metabolism in cancer, *British Journal of Cancer*, **122(2)**, 150-6 **(2020)**

19. Wishart D.S., Knox C., Guo A.C., Eisner R., Young N., Gautam B., Hau D.D., Psychogios N., Dong E., Bouatra S. and Mandal R., HMDB: a knowledgebase for the human metabolome, *Nucleic Acids Research*, **37(suppl_1)**, D603-10 **(2009)**

20. Wei J., Xie G., Zhou Z., Shi P., Qiu Y., Zheng X., Chen T., Su M., Zhao A. and Jia W., Salivary metabolite signatures of oral cancer and leukoplakia, *International Journal of Cancer*, **129(9)**, 2207-17 **(2011)**

21. Yakob M., Fuentes L., Wang M.B., Abemayor E. and Wong D.T., Salivary biomarkers for detection of oral squamous cell carcinoma - current state and recent advances, *Curr Oral Health Rep.*, **1(2)**, 133-141, doi: 10.1007/s40496-014-0014-y **(2014)**.

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