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EVALUATION OF SIALIC ACID, URIC ACID, TOTAL PROTEIN AND AMYLASE ACTIVITY IN BIOFLUIDS OF OSCC PATIENTS

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ABSTRACT: Background: Oral squamous cell carcinoma (OSCC) is one of the most prevalent cancers in developing countries like India. This high incidence and mortality rates result from unhealthy lifestyle, lack of awareness and late detection. Cancer progression has evolved to evade checkpoints governing normal functioning of a cell. This gives rise to biochemical signatures that can be indicators of tumour presence and progression. Exploring such variations in easily accessible biofluids for large scale screening and diagnostics is required. Methods: Fasting saliva, serum and urine samples from 25 OSCC patients and 50 healthy volunteers were collected and their clinico-pathological details noted. Sialic acid (Ehrlich method), total protein (Biuret method), uric acid (Uricase method) and amylase activity (Kinetic method) were measured in all samples. Shapiro–Wilk test, Mann-Whitney U test, Receiver Operator Characteristic curve analysis and Principle Component Analysis were used to analyze data. Results: The following changes were observed as compared to controls (i) Sialic acid in all three biofluids and total protein in saliva and urine were significantly higher (ii) Amylase activity was significantly lesser in serum and urine whereas uric acid was higher only in serum. ROC analyses and PCA of parameters showed that combination of sialic acid with total protein in saliva and urine, and sialic acid with uric acid in serum exhibited better differentiation between groups as compared to individual parameters. Conclusion: This study demonstrates the benefits of analysing multiple biochemical indicators over individual ones in non-invasive biofluids for improved differentiation of OSCC patients from healthy controls.

Keywords: OSCC, Biofluids, Sialic Acid, Uric Acid, Total Protein, Multivariate Analysis.

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1.INTRODUCTION

Cancer is a multifactorial disease with approximately 18.1 million new cases and close to 9.6 million deaths recorded globally in the year 2018 [1]. Asia alone contributes to 48.4% of this incidence and 57.3% deaths as reported by GLOBOCAN 2018. India, with a population of >1.3 billion, recorded ~1.2 million new cancer cases and 0.8 million cancer deaths in 2018 [1]. Among the most prevalent forms of cancers here, oral cancer is the second most occurring type and its incidence is higher in men as compared to women [1]. The survival rate of oral squamous cell carcinoma (OSCC), the commonest form of oral cancer, has remained close to 50% for 5 years [2]. Exposure to carcinogens from tobacco along with poor oral hygiene and late or poor prognosis have led to its high incidence in young adults and old-age population alike in developing countries [3]. Metabolomics, study of metabolic alterations, presents a gist of all genetic, epigenetic, proteomic and transcriptomic variations. This has thus augmented understanding of cancer progression as well as presented new prospects for diagnostic and treatment strategies that may complement existing methods [4-6]. However, application of such methods for mass screening in a developing country like India is economically not feasible. Hence a need still remains for easier non-invasive diagnostic strategies that may facilitate mass community screening, prognosis and monitoring of treatment progress in OSCC. Malignancy associated cellular metabolic changes alter composition of biofluids like serum, urine and saliva. Studying the viability of using such variations in designing diagnostic approaches has been garnering interest in the past decade [4]. Blood and urine are the preferred diagnostic media for many diseases including cancer [6-8]. In recent years, saliva is emerging as an alternate option for invasive diagnostic biofluids [4, 9, 10]. It has an added advantage, especially in OSCC, as the tumour vicinity fluid it may carry signatures of tumour tissue specific alterations. In the current study, measurements of sialic acid, total protein, amylase activity and uric acid have been carried out in saliva, serum and urine to examine their variations, if any, in OSCC patients from healthy controls. The thrust of this study is to choose biochemical parameters which can be easily analyzed in any diagnostic laboratory. An attempt to weigh the strength of such varying parameters, either individually or collectively, to distinguish OSCC patients from healthy controls has also been made.

2. MATERIALS AND METHODS

Subjects

This study has been approved by the Institutional Ethics Committee of St. John's National Academy of Health Sciences (SJNAHS) (IEC Study Ref No: 152/2014), Bengaluru and the Ethics Committee on Human Research of Bhagawan Mahaveer Jain Hospital (BMJH), (BMJH/ECHR/02/2013-14), Bengaluru. Samples were collected as per the methodology approved by these ethics committees. The study involved two groups; OSCC patients coded 'OC' and healthy controls coded 'HC'. A randomised sampling method was used. Patients in the pre-treatment phase, prior to definitive therapy from the Surgical Oncology Department, SJNAHS, Bengaluru and the Maxillofacial Department, BMJH, Bengaluru, comprised the OC group. People with recurrent malignancies, pregnant or lactating women, and minors were excluded from the study. Healthy subjects from comparable age/gender and socioeconomic status formed the HC group. All participants signed an informed written consent. Fasting samples of blood (≅3 mL) by venipuncture method, sputum free saliva ((≅3 mL) by expectoration method and first void urine (3-5 mL) were collected. Blood was collected in plain tubes without anticoagulant by the nursing staff in case of patients and a phlebotomist in case of healthy controls. All collected samples were immediately refrigerated and then transported to the University laboratory under appropriately chilled conditions. Sample tubes were centrifuged at 6000 rpm for 10 min at 4°C. Serum from blood and clear supernatant from saliva and urine were freeze preserved at −80°C till analysis. Frozen samples were thawed on ice. Serum, saliva and urine concentration of sialic acid was determined by Ehrlich method [11], uric acid by Uricase method using Infinite Uric Acid Liquid kit, Accurex Pvt Ltd, Mumbai, India [12], total protein by Biuret method using Autozyme Total Protein Biuret Kit, Accurex Pvt Ltd [13], and amylase activity by Kinetic method using Infinite α-AMYLASE KINETIC kit, Accurex Pvt Ltd [14].

Statistical analysis

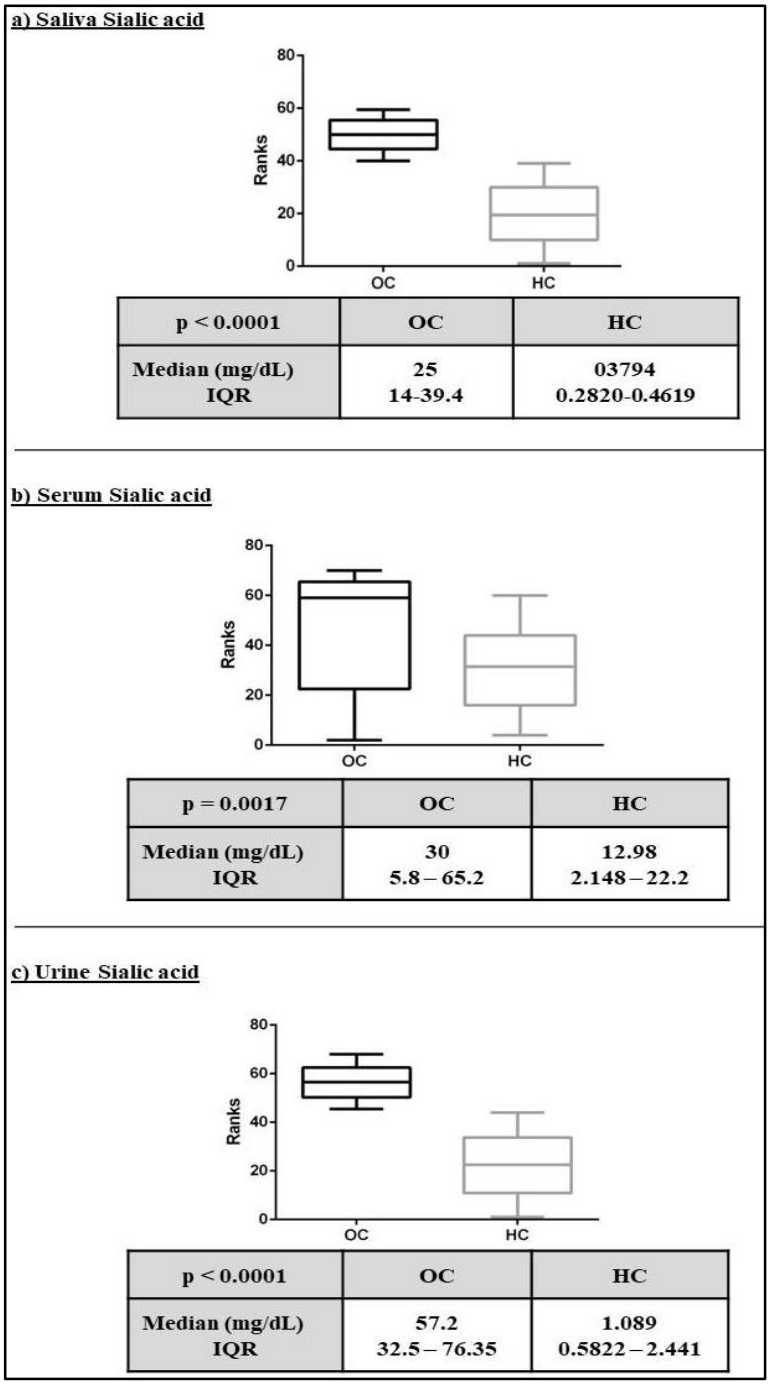
GraphPad Prism 6 (GraphPad Software, Inc., San Diego, California, US) was used to perform descriptive analyses. Non-detects in data were replaced by half of the detection limit of respective analyses. Shapiro–Wilk test was performed to examine data normality. Mann-Whitney U test was performed to check equality of mean ranks between the two study groups in non-normal data. Outliers were eliminated and results with $p<0.05$ and confidence levels of 95% were considered significant. Parameters that showed significant variation between the two study groups were subjected to Receiver Operator Characteristic (ROC) curve analysis using XLSTAT (Addinsoft SARL, Long Island City NY, US). Parameters that expressed acceptable sensitivity and specificity were grouped biofluid-wise and subjected to ROC curve analysis and Principle Component Analysis (PCA). Optimum cut-off values for these parameters were identified based on best likelihood ratio from ROC curve using GraphPad Prism 6.

3. RESULTS AND DISCUSSION

Table 1: Characteristics of study groups

Particulars	OSCC patients	Healthy controls
Number		
Total ……..	25	50
Female. ……..	10	19
Male.…….	15	31
Male: Female.……	1.5	1.6
Age (years)		
Male + Female: mean (range) ……	54.24 (33-76)	53.78 (41-69)
Male: mean (range)…….……	50 (33-76)	46 (41-69)
Female: mean (range) ……...……	60.5 (49-67)	43 (42-69)
Squamous cell carcinoma site		
Buccal mucosa ……	12	-
Tongue ……	7	-
Gingiva ……..……	3	-
Floor of Mouth ……..	3	-
Histopathological differentiation		
Well …….	21	-
Moderate …….	4	-
Clinical stage of the disease (TNM)		
Stage I …….	3	-
Stage II.…….……	4	-
Early stage (stages I + II) ……	7	-
Stage III.……	2	-
Stage IV……	16	-
Advanced stage (stages III + IV) …….……	18	-
Lymph node metastasis	20	-
Type of tobacco habits		
Chewers …….	12	-
Smokers …….	5	-
Alcohol …….	7	-
Betel leaf with tobacco …….	7	-
Chewers + smokers ……	3	-
Tobacco + alcohol ……..	6	-

A total of 30 OCs and 50 HCs were enrolled in this study. Samples from five patients were not analyzed as four were not collected in accordance to protocol and one patient had multiple co-morbid conditions. Twenty-five OC and 50 HC samples were analyzed for earlier mentioned parameters. This sample size was calculated based on previous literature findings, with 90% statistical power and 5% level of significance. A questionnaire was filled by referring to the hospital patient chart as well as by interviewing each participant (Table 1). Confidentiality was maintained with respect to participants' personal details. Patients were coded from P1 to P30 and healthy controls as HC1 to HC50. Comparative analysis of sialic acid, total protein, amylase activity and uric acid levels in serum, saliva and urine of OC and HC groups was carried out. Shapiro–Wilk test results showed that most data was non-normally distributed. Hence non-parametric analysis, Mann-Whitney U Test, was carried out and results were reported as median and interquartile range (IQR). Sialic acid showed significant increase in all three biofluids in OC group (Figure 1). Total protein was significantly higher in saliva and urine of OC samples (Figure 2 a & b) but showed no difference in serum [OC: 6.121 (5.371 – 7.57); HC: 6.746 (6.402 – 7.394); $p = 0.1395^{ns}$] between both groups. Amylase activity was significantly reduced in serum and urine of OC patients (Figure 2 c & d). However, no significant variation was observed in salivary amylase activity levels between OC [20243 (2436 – 34305) IU/L] and HC [31235 (11634 - 41305) IU/L] samples. Significant increase in serum uric acid levels was seen in OCs (Figure 3) with no changes in saliva and urine between the two study groups. A breakup of number of samples in HC and OC groups that presented low, normal and high uric acid levels has been presented in Table 2. No significant variation was observed in salivary [OC: 3.664 (1.5 – 5.419); HC: 3.665 (2.043 – 6.331) mg/dL; $p = 0.6232^{ns}$] and urinary [OC: 12.72 (1.5 – 5.419); HC: 3.665 (2.043 – 6.331) mg/dL; $p = 0.6232^{ns}$] sialic acid levels.

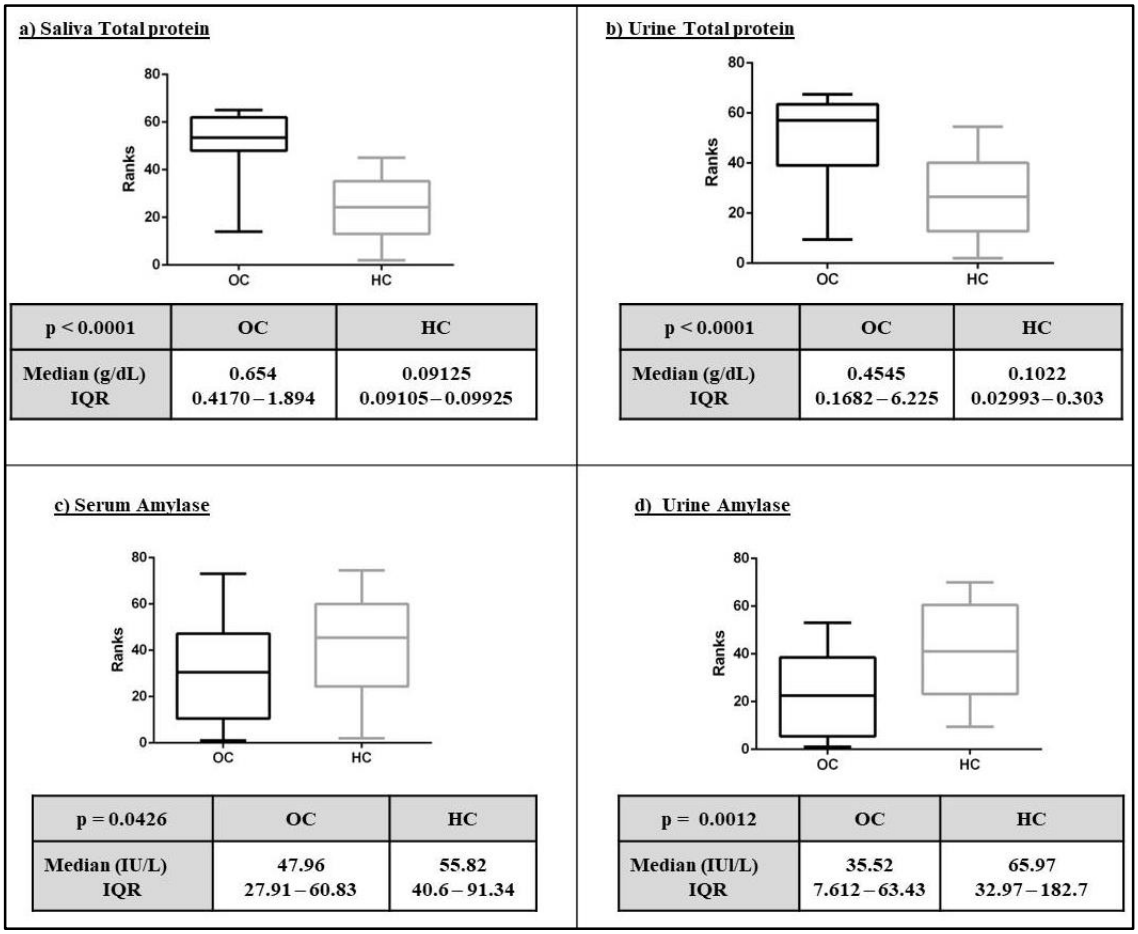


p < 0.0001	OC	HC
Median (mg/dL) IQR	25 14-39.4	03794 0.2820-0.4619

p = 0.0017	OC	HC
Median (mg/dL) IQR	30 5.8 – 65.2	12.98 2.148 – 22.2

p < 0.0001	OC	HC
Median (mg/dL) IQR	57.2 32.5 – 76.35	1.089 0.5822 – 2.441

Figure 1: Descriptive analysis and Mann-Whitney U Test results of Sialic acid (a) Saliva (b) Serum (c) Urine in OSCC patients' (OC) and healthy controls (HC) samples



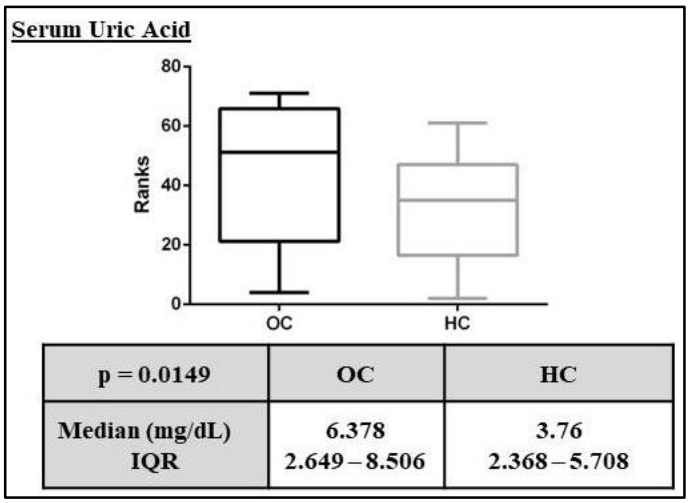
p < 0.0001	OC	HC
Median (g/dL) IQR	0.654 0.4170 – 1.894	0.09125 0.09105 – 0.09925

p < 0.0001	OC	HC
Median (g/dL) IQR	0.4545 0.1682 – 6.225	0.1022 0.02993 – 0.303

p = 0.0426	OC	HC
Median (IU/L) IQR	47.96 27.91 – 60.83	55.82 40.6 – 91.34

p = 0.0012	OC	HC
Median (IUl/L) IQR	35.52 7.612 – 63.43	65.97 32.97 – 182.7

Figure 2: Descriptive analysis and Mann-Whitney U Test results of (a) Saliva total protein (b) Urine total protein (c) Serum amylase activity (d) Urine amylase activity in patients' (OC) and healthy (HC) samples



p = 0.0149	OC	HC
Median (mg/dL) IQR	6.378 2.649 – 8.506	3.76 2.368 – 5.708

Figure 3: Descriptive analysis and Mann-Whitney U Test results of Serum uric acid in patient (OC) and healthy (HC) samples

Table 2: Serum Uric acid levels in patients' and healthy samples

Uric acid levels	OSCC patients	Healthy Controls
Low (<3 mg/dL)	8 (33.33 %)	18 (38.297 %)
Normal (3-6 mg/dL)	2 (8.33 %)	21 (44.68 %)
High (6 mg/dL)	14 (58.33 %)	8 (17.0218 %)
Total	24	47

Of the four measured parameters, two in saliva, three in serum and three in urine showed significant variations. These parameters were individually subjected to ROC curve analyses and resulting sensitivities, specificities and AUC (Area Under Curve) values are presented in Table 3. Sialic acid and total protein in saliva and urine; sialic acid and uric acid in serum showed relatively better sensitivity and specificity and were further combined for PCA and ROC curve analysis. Optimal cut-off values for each of these parameters that could predict the possible occurrence of malignancy have been compiled in Table 4.

Table 3: Results from ROC curve analysis of parameters individually and in combinations

Parameter	Sensitivity	Specificity	AUC
Saliva Sialic Acid	48.28%	78%	0.783
Saliva Total Protein	48.28%	100%	0.806
Saliva Sialic acid and Total protein	65.52%	100%	1
Urine Sialic Acid	58.62%	100%	0.928
Urine Total Protein	24.14%	88%	0.664
Urine Sialic acid and Total protein	62.07%	100%	0.994
Serum Sialic Acid	27.59%	98%	0.73
Serum Uric Acid	34.48%	94%	0.668
Serum Sialic Acid and Uric Acid	62.07%	100%	0.994

*AUC: Area Under Curve

Table 4: Cut off values that differentiate OCs from HCs

	Cut-off	Sensitivity	Specificity
Salivary Sialic acid	5.4 mg/dL	95.24%	100%
Salivary Total protein	0.1436 g/dL	86.96%	97.62%
Serum Sialic acid	6.046 mg/dL	58.33 %	82.98 %
Serum Uric Acid	20.75 mg/dL	71.43 %	75.51 %
Urine Sialic acid	13.5 mg/dL	91.67 %	100 %
Urine Total protein	0.1723 g/dL	72%	79.55 %

Figure 4 A, B, C presents the PCA score plots for saliva, serum and urine respectively. F1 represents the first principal component representing highest variance and F2 the second principal component with second highest variance. The combined variance captured by F1 and F2 is proportional to the combined differential capacity of parameters involved. The tight clustering of healthy samples (blue dots) demonstrated similarly within the group, whereas scattering of patient samples (green dots) demonstrates the extent of variability present in this group.

DISCUSSION

Tumour formation and progression is accompanied by a consortium of disruptions in normal regulation of various pathways leading to changes in various metabolites. Identification and validation of such changes, that may be easily measurable in non-invasively accessible media, will complement existing screening and diagnostic methods. In this study, four biochemical parameters namely sialic acid, total protein, amylase activity and uric acid were chosen and estimated in saliva, serum and urine to differentiate OSCC patients from normal.

Sialic acids, cell surface molecules capable of reflecting changes in cell membranes, are synthesized in golgi-complex and attached as glycan end moieties to cell membrane proteins and lipids [15, 16]. In normal cells, sialic acids are multi-functional owing to their structure and distribution on the cell membrane which maintains cell integrity [7]. Normal functioning of the cell stands disrupted in malignant transformations which also involves higher sialylation that enhances cell-cell repulsion due to the negative charge present on sialic acid moieties and helps in invasion / metastasis by masking the antigenic sites on tumour cells [9]. An altered sialylation pattern has been observed in cancer derived immunoglobulins and liver synthesized positive acute phase proteins such as haptoglobin, α-1-antitrypsin and α-1-acid glycoprotein as well [7]. Studies have reported an escalation in sialic acid levels in the premalignant phase as well as in relation to the disease grade in OC [17, 18]. In addition, an enhanced sialyltransferase activity, reduced sialidase activity and/or increased sialylglycoprotein production have also been observed during tumour progression [7]. Secretion and shedding of sialic acids due to high turnover of cells results in an enhanced sialic acid content in biofluids [18]. In this study a significant increase in sialic acid levels was observed in all three biofluids in the OC group. Similar increase has been reported by earlier studies in OSCC patients' sialic acid in serum [18] and in saliva [9, 19, 20]. Reported serum values of sialic acid in healthy volunteers varied over a range from ≈8 to ≈63 mg/ dL [15, 18, 21, 22]. Serum values in the present study (median: 12.98 mg/dL) are close to those reported by Dadhich, Prabhu [15] (mean ± SD: 7.515 ± 6.86 mg/dL). Saliva HC values (median: 0.38 mg/dL) fall close to the range reported by Dahal, Boaz [23] (1 – 4.90 mg/dL) and urinary values (median: 1.089 mg/dL) to that reported by Nayak and Bhaktha [24] (3.2 ± 0.65 mg/dL). Earlier studies in OC have reported >60 mg/dL in serum and saliva, however the present study has recorded a smaller range (Figure 1). These variations across studies may be due to difference in

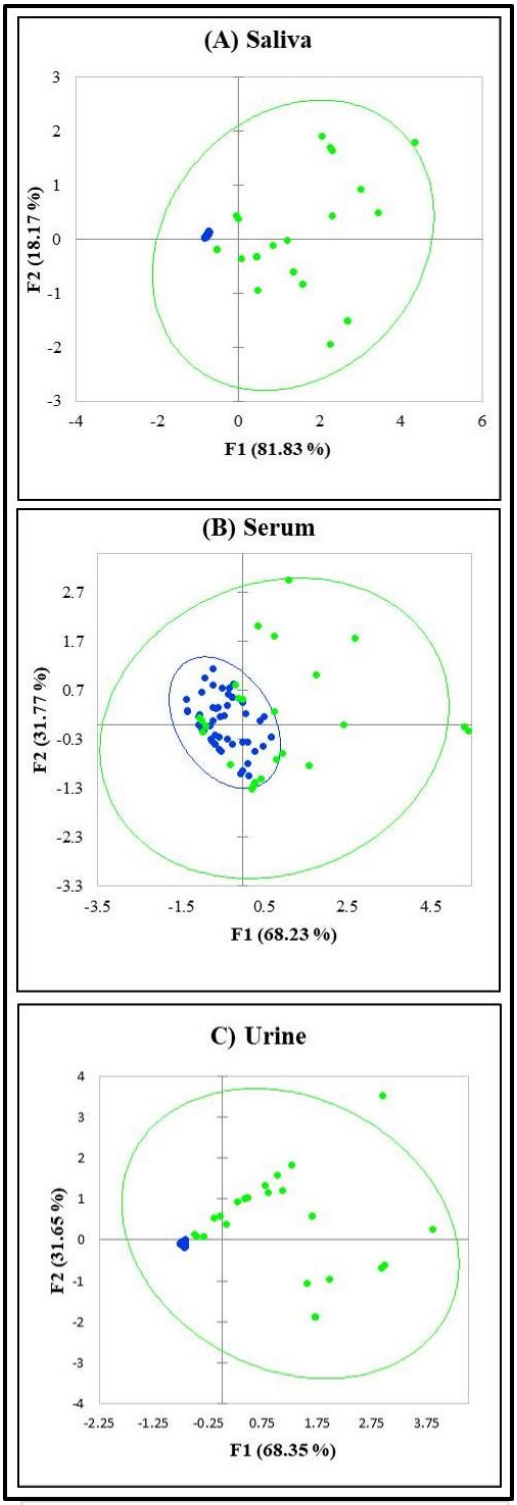


Figure 4: PCA score plots (A) Salivary sialic acid total protein (B) Serum sialic acid and uric acid (C) Urine sialic acid and total protein. Blue dots: healthy controls; Green dots: OSCC patients

sample collection and analytical methods; hence each laboratory may have to establish its own reference range. Sialic acids are degraded in lysosomes and excreted via urine [16]. Thus, an increased serum sialic acid concentration may be reflected in patients' urine as seen in this study. Proteins form structural, enzymatic and immunologic components of a biological system. Occurrence of malignancy may cause measurable variations in the overall protein content. In this study, total protein levels observed in HC group (Figure 2 a & b) are comparable to previously reported reference ranges in serum (5.6 mg/dL), saliva (0.47 µg/L) and urine (≈ 0.05 mg/mL) [13, 25, 26]. Salivary and urine total protein levels were found to be higher in patients whereas protein levels in serum were similar in both study groups. Earlier studies on serum from OSCC patients have reported both a decrease [6, 27] and an increase [2, 8] in protein concentration. A decrease in protein levels is a sign of catabolic state where cells are actively broken down either to suffice the growing nutrient need of proliferating tumour cells or due to an enhanced oxidative damage [6, 27]. On the contrary, an increase may be observed due to an augmentation of cell mass accompanied with production of positive acute phase reactants [8]. Previous studies on salivary content in oral cancer patients have reported an enhanced protein concentration similar to this study [6, 8, 19]. It has been suggested that aberration to the mucosal lining or capillaries in oral cavity, antioxidants/ antimicrobial proteins produced in response to inflammation or tumors, draining of serum proteins etc., can contribute to such enhancement of salivary protein levels [6, 8]. It may be noted that none of the participants in HC or OC group suffered from any other condition that may cause variation in urine protein content. Therefore, the observed increase in protein concentration in OC may be attributed to the presence of OSCC in this study. Amylase is a digestive enzyme that hydrolyses starch to maltose. About 40% of amylase in the body is made by the pancreas and the rest by salivary glands. Salivary α-amylase/ptyalin accounts for almost 40-50% of total salivary protein content and aids in digestion of ingested food starting from the mouth up till stomach. Pancreatic amylase facilitates digestion in the small intestine and further. Therefore, any change in salivary amylase activity can be detected in saliva without interference from pancreatic amylase. Variations in salivary amylase can also be deduced in serum in the absence of any known pancreatic anomalies affecting pancreatic amylase levels. Amylase is one of the very few enzymes that get filtered from blood into urine with no passive secretion or re-absorption, thereby reflecting overall amylase level variations [28-31]. Amylase activity in this study was measured in all three biofluids. Normal samples' values observed in this study (Figure 2 c & d) fall closer to the reported range in serum (40-140 U/L) [32] saliva (113-1125 U/mL) [14] and urine (2.6 – 21.2 IU/h) [33]. A significantly reduced activity was observed in both serum and urine of OC group, whereas salivary amylase levels did not exhibit any significant variations. Uppala [34] also did not observe any variation in amylase activities between the OSCC patients' and healthy samples. Whereas Awasthi [2] observed a reduced salivary amylase activity in oral cancer patients, which they attributed to low salivary flow rate as a consequence of

compromised oral environment and altered concentration of various enzymes, ions, and electrolytes in saliva. Khan [10] suggested that injuries inflicted to the ductal secretory units and a dilution due to an increased salivary flow in tobacco smokers and chewers can contribute to a decreased amylase activity. A compromised epithelium, made more permeable to toxics from tobacco, is also thought to contribute to variations in the biochemical composition of saliva [10]. Studies in serum and urine amylase levels in OSCC are scarce; hence a comparative analysis of this variable could not be carried out. The reduced amylase activity observed in this study may be associated with salivary gland activity which may be reflected in serum and urine. This study observed significantly high serum uric acid levels in patients' samples than HCs whereas uric acid levels in saliva and urine were similar in both study groups. The increase in serum UA levels observed here is majorly contributed by 58% of high UA expressing samples in the patient group (Table 2). Similarly, an increase in serum uric acid levels in a subgroup of studied OSCC samples was also reported by Sharma [5]. An association of increased serum uric acid levels with a higher cancer risk attributed to a high turnover of cells in tumours, has also been reported [35]. Conversely, Kolude [36] reported lower serum uric acid levels to be associated with an increased oral cancer risk. Nosratzehi [37] examined saliva in OSCC and found no variations in UA levels between patients and healthy controls. In contrast, and Demeri [12] reported lesser salivary uric acid levels in OSCC patients in comparison to healthy controls. It is known that, uric acid in hydrophilic environment, acts as a chain breaking antioxidant molecule that scavenges hydroxyl, superoxide, peroxyl and peroxynitrite radicals thus preventing lipid peroxidation in cellular membrane [5]. UA contributes to approximately 70% antioxidant activity in saliva and close to 60% in serum [12, 39]. In a hydrophobic environment it is known to interact with other pro-oxidants and contribute towards higher lipid peroxidation [5, 40]. Recently, Yun [41] examined different organs in rats for correlations between serum uric acid levels and genes specific to apoptosis and cell regeneration. They showed that nucleosides, released from nucleus and cytoplasm of dying cells, degrade and result in purines that get recycled partly and the rest are converted to uric acid. This uric acid is transported to liver from other organs, via blood, for further degradation. Hence an increase in serum uric acid level can be a specific but less sensitive indicator of cell death owing to tumour induced cell necrosis. Unlike the earlier suggested notion regarding the antioxidant role played by uric acid, they did not observe any relation between uric acid levels and production of ROS in organs with high metabolic rates. Parameters that varied significantly between the two study groups were analysed for their diagnostic / prognostic potential in OSCC using ROC curve analysis. From this single parameter analysis, those biomolecules with better sensitivity, specificity and AUC values were grouped in each biofluid. Sialic acid and total protein in both saliva and urine; sialic acid and uric acid in serum were found to be better differential parameters in the current study. Comparison between single ROC curve results and those derived from grouped variables shows that the ROC curve output improved in combination (Table 3). In

order to explore the intrinsic variability and differential clustering capacity of these combinations, PCA was also performed (Figure 4). Improvement in the AUC values and noticeable differential clustering of HC and OC groups indicate that the option of analyzing multiple parameters serve better than a single factor based one. Based on similar studies, Franzmann and Donovan [4] have developed a point-of-care dip-strip device for accessing CD44 transmembrane protein and total protein in oral washes as indicators to occurrence of oral malignancies.

4. CONCLUSION

The present study observed a significant increase in sialic acid, total protein and uric acid levels in biofluids of OSCC patients. These variations can be attributed to the presence of OSCC alone as the patients here were known to be free from any other conditions. Differential capacity of sialic acids (indicators of cell surface modifications) improved in combination with uric acid (cell death indicator) in serum and with total protein in saliva & urine. Such analyses of multiple parameters across biofluids may facilitate interpretation of variations as metabolic signatures caused by malignancy. Further studies based on these observations may be carried out aimed at enhancing the robustness of existing diagnostic or treatment management procedure.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Institutional Ethics Committee of St. John's National Academy of Health Sciences, (IEC Study Ref No: 152/2014), Bengaluru.

Ethics Committee on Human Research of Bhagawan Mahaveer Jain Hospital, (BMJH/ECHR/02/2013-14), Bengaluru.

HUMAN AND ANIMAL RIGHTS

Humans saliva, serum and urine samples were used for this study after obtaining necessary ethical approvals and volunteer consent.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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