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COMPARITIVE EVALUATION OF LDH ENZYME ACTIVITY IN SERUM & SALIVA OF OSCC & OSMF PATIENTS

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ABSTRACT:

Background : Oral squamous cell carcinoma (OSCC) encompasses about 90% of all oral malignancies. India tops in prevalence of oral cancer in the world and it remains the commonest cancer among male population & the third-most common cancer of women in India . Potentially malignant lesions of oral cavity are relatively common, occurring in about 2.5% of the population with a malignant transformation rate that ranges from 0.6 to 20% .The malignant transformation involves glycolytic pathways that can alter lactate dehydrogenase(LDH) levels . As the profile of salivary LDH is similar to that found in oral epithelium, LDH concentration in saliva, can be used for evaluating oral mucosal changes even during malignant transformation.

This study was an attempt done to find a standard range of Salivary LDH levels in controls & compare the same with saliva LDH levels of patients of OSMF and OSCC as also with serum LDH levels of the same patients.

Materials & Method: 75 Subjects in the age group of 18–60 years - 25 diagnosed with oral submucous fibrosis(OSMF),25 with OSCC & 25 controls were included. Pooled Saliva was collected by spitting, centrifuged at 2500 rpm for 15 min & analysed for LDH with BA kit using semiautomatic analyser . Serum was also similarly analysed. The data obtained was subjected to statistical analysis using the SPSS software version 17.

Result & Conclusion: We found a statistically significant increase in both serum & saliva LDH in OSMF & OSCC patients as compared to controls. The comparison of salivary & serum LDH levels between OSMF / OSCC cases and healthy subjects was statistically significant S($P<0.001$)

Thus, clinical diagnosis supplemented with LDH levels can gain diagnostic importance in near future & serve as a valuable aid in monitoring treatment outcomes in the OSMF & OSCC patients.

Keywords: OSCC, OSMF ,Tumor markers, LDH, Saliva, Warburg effect

INTRODUCTION:

Oral squamous cell carcinoma (OSCC) encompasses at least 90% of all oral malignancies. It is recognized to have 50%, five year survival rate. In India, the incidence of oral cancer is about three to seven times more as compared to developed countries & it accounts for approximately 1/3rd global burden of this disease. India tops in prevalence of oral cancer in the world and oral cancer remains the commonest cancer among male population. Among women, Oral cancer is the third-most common cancer in India after cervical and breast cancer.[1,2,3]

Potentially malignant lesions of oral cavity are relatively common occurring in about 2.5% of the population [5], with a malignant transformation rate in various studies & locations that range from 0.6 to 20%.[4] Oral leukoplakia is the most commonly occurring precancerous lesion

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of the oral cavity representing 85% of such lesions [6].

Potentially Malignant Disorders Epithelial Precursor Lesions are described as lesions which may have an increased potential for malignant transformations. Oral Submucous Fibrosis is a potentially malignant disorder and a debilitating condition of the oral mucosa [8]. It has also been found that Indian patients with oral submucous fibrosis have a higher risk in developing carcinomas than those without this disease. Clinically visible lesions are non- cancerous to begin and have been present for a varying length of time.

Oral Submucosal Fibrosis is an insidious, chronic disease affecting any part of the oral cavity and sometimes the pharynx [4].Occasionally it is preceded by and/or associated with vesicle formation [5] and is always associated with juxta-epithilial inflammatory reaction followed by progressive hyalinization of lamina propria [6]. Then, later subepithelial and submucosal myofibrosis leads to stiffness of the oral mucosa and deeper tissues with progressive limitation in the opening of the mouth and prostrusion of the tongue, thus causing difficulty in eating, swallowing and phonation [7].

There is therefore, a mounting concern to recognize this dreadful disease in its early stages. Early detection is the key for effectual management of oral submucous fibrosis, better prognosis and helps to reduce mortality and morbidity of such cases.OSMF is having a prevalence rate of 6.42% in India.The malignant potential of OSMF ranges from 4.5% to 7.6% [11].Among all the etiological factors of OSMF,betel nut & areca nut play a key role.

The high incidence of oral cancer in India has also been linked with habits of tobacco chewing &smoking. Carcinogenic changes in mucosa are usually a result of consistent & /or longterm exposure of mucosal epithelium to exogenous &/or endogenous precarcinogens ,carcinogens and suppression of anticarcinogens ,tumor suppressors.

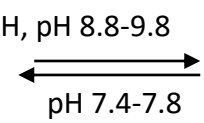
The early diagnosis and treatment of cancer are based on the concept that oral cancer develops over long period of time, going through intermediate stages of different biological significance. Treatment at this early or pre-invasive stages offer the best prognosis and even the chances of cure. Therefore, if precancerous lesions are detected and treated early then the conversion to cancer is averted. Despite the advances made in the therapeutic modalities via multidisciplinary approaches, survival rate for OSCC has not significantly improved.[7] This motivates the search of factors which will help in the early diagnosis and management. Early diagnosis and prompt treatment will avert mutilating surgery, improve patients quality of life and can decrease morbidity and mortality associated with cancer.

Tumor markers are diagnostic aids that play a very important role in early diagnosis and interception of cancer. Tumor markers in serum, tissue and other body fluids during neoplastic process are of clinical value in the management of patients with various body cancers. Among all the body fluids, blood has been the media of choice for the study of the biochemical markers by the medical community but it does have some inherent disadvantages. Collecting blood for investigation is an invasive procedure and has a potential risk of disease transmission through needle stick injuries. Despite the absence of charisma, however, a growing number of researchers are finding that saliva provides an easily available, non-invasive diagnostic medium for rapidly widening range of disease and clinical situations.[9]

Saliva is a complex and dynamic biologic fluid, which over the years has been recognized for the numerous functions it performs in the oral cavity. Modern technology, however, has unveiled a plethora of compounds never before detected in saliva like drugs, pollutants, hormones and also biomarkers of bacterial, viral, and systemic diseases. Saliva based diagnostics are more attractive as they are more accessible, accurate, less expensive and presents less risk of infection to the patient, health care worker and cross infection. With all these above mentioned added advantages saliva can serve as diagnostic tool as compared to serum.[10]

LDH:

LDH is an oxidoreductase that catalyzes the interconversion of pyruvate to lactate.



The profile of salivary LDH is similar to that found in oral epithelium, indicating that the major source of salivary LDH is probably the oral epithelium-shedding cells.[10] The similarity between the profile of LDH in whole saliva and the oral epithelium supports the hypothesis that salivary LDH is predominantly of extra glandular origin. Consequently, LDH concentration in saliva, as an expression of cellular necrosis, could be a specific indicator for oral lesions that affect the integrity of the oral mucosa. Thus, salivary LDH levels may be evaluated for possible oral mucosal pathologies.[11] as they could play a significant role in the diagnosis of pathologic process & also serve as a disincentive in continuing the habit.

Tissue LDH levels are very high (500 times) as compared to its serum levels. Therefore, leakage of the enzyme from even small amount of damaged tissue can increase LDH levels to significant levels so, substantiating its significance

as a biomarker of tissue damage in saliva.

The enzyme lactate dehydrogenase (LDH) is found in the cells of almost all body tissues. It is especially concentrated in the heart, liver, red blood cells, kidneys, muscles, brain, and lungs. Increased serum LDH activity is considered as a marker of cellular necrosis and serum LDH levels have been used as a biochemical marker in diagnosis in various cancers like oral, laryngeal and breast cancer. LDH activity is mainly due to genomic changes during malignant transformation. Increased LDH levels are due to increased mitotic index leading to more lactic acid production by tumor cells due to breakdown of glycoprotein.(9)As the magnitude of dysplastic changes increase in leukoplakia , OSMF or OSCC it is logical to expect increase in values of LDH.

The LDH activity in OSMF cases can be related to the muscle fatigue also,due to chewing that might eventually increase the glycolytic activity. Muscle fatigue causes accumulation of pyruvate due to hypoxia that has to be converted to lactate, resulting in high glycolytic activity in OSMF. With increase in glycolytic activity, concomitant increase in lactate dehydrogenase enzyme may be reflected in some tissues.[12]

Another reason is the hypoxic state related to OSMF. Hypoxia triggers glycolytic pathways. In hypoxic state or in absence of oxygen, the pyruvate, the end product of glycolysis, is converted to lactate. This reaction is mediated by LDH [10]. So in these conditions by reflex LDH levels are increased. Tilakarathne et al. [13], showed that increased hypoxia plays a role in malignant transformation and progression of OSMF.

Warburg Effect & Effect of Lactate on Tumor Microenvironment:[15,16,17]

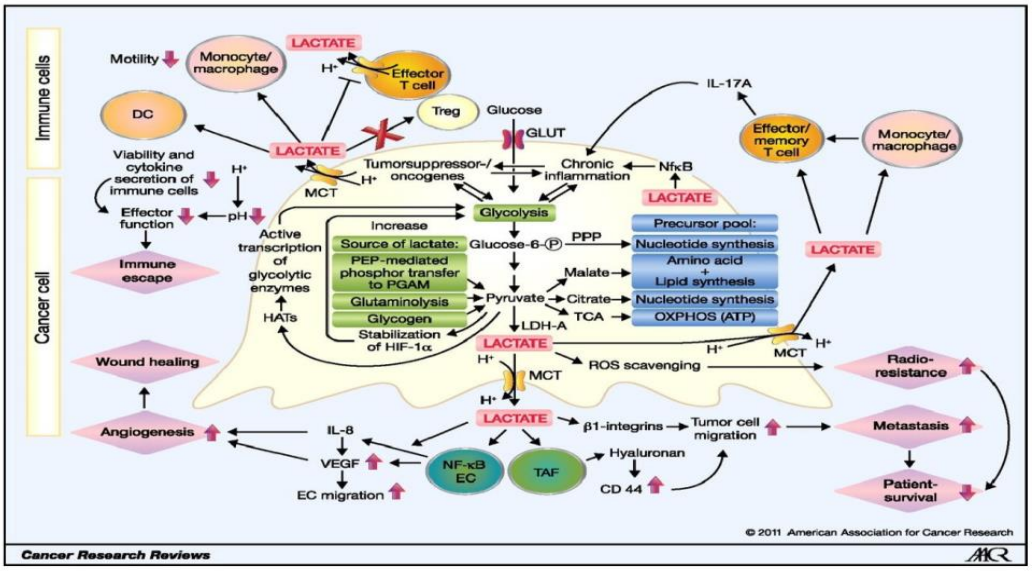
In1927, Otto Warburg described that, metabolically, tumor cells predominantly rely on increased glycolysis, followed by lactic acid fermentation, even under conditions where oxygen is available. This process was dubbed by him as “aerobic glycolysis. By contrast, untransformed epithelial cells produce about 20% of their daily energy from glycolysis whereas the rest (about 70%) of that energy comes from the Krebs cycle.

Metabolic reprograming of tumor cells modifies the metabolic fluxes, restructuring the Krebs cycle and enhancing glycolysis. The enhanced glycolytic carbon flux, in turn, leads to production of high amounts of lactic acid. It is estimated that tumor cells produce up to 40 times more lactic acid than normal cells .

Thus, Malignant transformation of cells leads to enhanced glucose uptake and the conversion of a larger fraction of pyruvate into lactate, even under normoxic conditions by the Warburg effect. This metabolic reprograming serves to generate biosynthetic precursors, thus facilitating the survival of rapidly proliferating malignant cells.

The tumor microenvironment is an intricate network of extracellular matrix molecules, soluble factors and cells, including stromal cells and adipocytes. Tumor stromal cells include cancer-associated fibroblasts (CAFs), tumor endothelial cells (TECs), and immune inflammatory cells such as macrophages. Stromal cells generate a tumor microenvironment in constant change as tumors invade normal tissues and subsequently seed and metastasize. Among the soluble factors present in the tumor microenvironment, lactate is of particular importance given its effects on cancer and stromal cells.

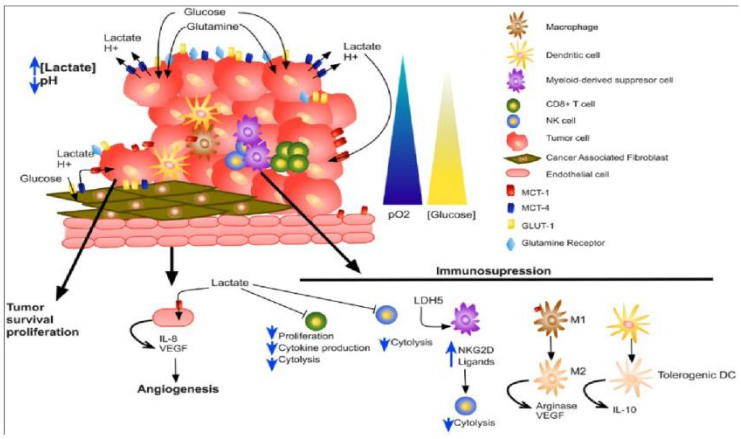
Fig-1: Illustration of lactate as a key player in cancer. DC, dendritic cell; EC, endothelial cell; GLUT, glucose transporter; IL, interleukin; HAT, histone acetylase; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; PEP, phosphoenolpyruvate; PGAM, phosphoglycerate mutase; PPP, pentose phosphate pathway; ROS, reactive oxygen species; TAF, tumor-associated fibroblast.



By the Warburg effect, cancer cells secrete large amounts of lactate to the extracellular microenvironment, which in turn lowers extracellular pH to 6.0-6.5. Lactate contributes to acidosis, signals for angiogenesis, acts as a cancer cell metabolic fuel, and induces immunosuppression. Several reports demonstrate that acidosis leads to loss of the T-cell function of human and murine tumor-infiltrating lymphocytes.The T-cell function can be restored only by buffering the pH at physiological values. In addition, stromal cells participate in a Reverse Warburg Effect, where fibroblasts may produce lactate that normoxic tumor cells consume to produce energy. Moreover, several tumor-infiltrating immune cells contribute, to some extent, to the total

amount of lactate within the tumor. Lactate is not only consumed by tumor cells for their survival, but it also stimulates angiogenesis. What is moreimportant is that, lactate has an immunosuppressive role, affecting several immune cell functions such as T-cell proliferation, cytokine production, and cytotoxic activity of NK and $CD8^+$ T cells (Figure 2).

Fig-2: Impact of lactate on tumor microenvironment. Increased lactate secretion by tumor and stromal cells acidifies the tumor microenvironment, increases tumor cell survival and proliferation, stimulates angiogenesis, and results in skewed immune response by altering several immune infiltrating cells.



Thus, the possible role of lactate as a predictive biomarker of overall survival of cancer patients arises from several studies indicating that lactate intra-tumoral levels are inversely correlated with overall and disease-free patient survival, as reviewed by Hirschhaeuser et al. [16]. Similarly, Blatt et al. [17] showed that, in a cohort of head and neck squamous cell carcinoma patients, high lactate levels in tumor tissue were inversely correlated with the overall and recurrence-free survival after surgery and radiation during a 15-year follow-up.

Role of Dental Surgeons:

Dentists, in particular, the Oral Physicians, are most often the first clinician consulted for general oral complaints and in an ideal situation they regularly screen patient's oral mucosa for early signs of oral cancer or precancer. He or She has the unique opportunity in the routine mouth examination to detect malignant neoplasm while they are still asymptomatic, innocuous and unsuspected and therefore has the critical responsibility of differentiating benign from precancerous and malignant conditions.

The detection and appropriate treatment at the level of premalignancy can greatly reduce the changes of further development into oral cancer, especially if the tobacco-chewing habit is discontinued. Early detection can also be a key to reducing the mortality,

morbidity and reducing the cost of treatmentThe focus of early detection has shifted to advanced biomedical techniques, such as identification of molecular markers that will allow staging of tissue change before changes in cell morphology. More important however are, Alternative techniques, which are monetarily more feasible for a rural population, & therefore should be explored. For this reason, the application of a simple Salivary screening &/or bloodscreening test using a well-known molecular marker, lactate dehydrogenase (LDH),can be employed.

Serum and Salivary LDH levels have not been studied rigorously in oral precancer and cancer. Hence, our study is an endeavor to measure, & attempt to find a standard range of Salivary LDH levels in controls .Subsequently ,we also compared & analysed the same with saliva LDH levels of patients of OSMF and oral squamous cell carcinoma . We also tried to evaluate if, salivary estimation of LDH can substitute serum LDH estimation in all three groups.

MATERIALS AND METHODS:

Source of data: Patients were selected from those attending the Out Patient Department (OPD) of Oral Pathology, GDCH, Raipur and patients were divided into three groups as follows:

Study Groups:

- Group I: Normal healthy individuals: 25 individuals
- Group II: Patients with OSMF: 25 individuals
- Group III: Patients with Oral Cancer: 25 individuals

Total Sample Size: 75 individuals

Patient selection Criteria:

Subjects in the age group of 18–60 years and diagnosed with oral submucous fibrosis or OSCC were included.

Controls :

Individuals in the age group of 18–60 years without any systemic or oral mucosa pathology. Exclusion criteria were same as for patients.

Exclusion criteria:

1. Subjects with history of systemic diseases and disorders that alter the levels of lactate dehydrogenase (myocardial infarction, pulmonary disorders, renal pathologies, hepatic failure),

2.Subjects with history of AIDS, bleeding dyscrasias, and

3.Subjects under corticoster-oids for any systemic disease were excluded.

4. Subjects previously treated for OSCC

Blood collection:

Informed written consent was obtained from the patients,following which,Venous blood measuring 2 ml was collected from each patient ,centrifuged & was evaluated for LDH levels using the standard kit method with semi

automatic biochemical analyser. LDH activity in serum is proportional to the increase in absorbance due to the reduction of NAD.

Saliva Collection & Analysis:

Those patients from whom venous blood was taken ,were made to clean their mouth & subsequently instructed to pool their saliva in the mouth without spitting or swallowing for 5 min & sitting in a relaxed posture. After that, 5 ml of unstimulated whole saliva was aseptically collected in a wide mouthed container ,by spitting without force or suction by the patient. Following the collection , saliva was centrifuged at 2500 rpm for 15 minutes and then it was analysed by ERBA CHEM 5 semi auto analyser for LDH. The BA kit works on the principle that LDH catalyzes the oxidation of lactate to pyruvate accompanied by the simultaneous reduction of NAD to NADH.

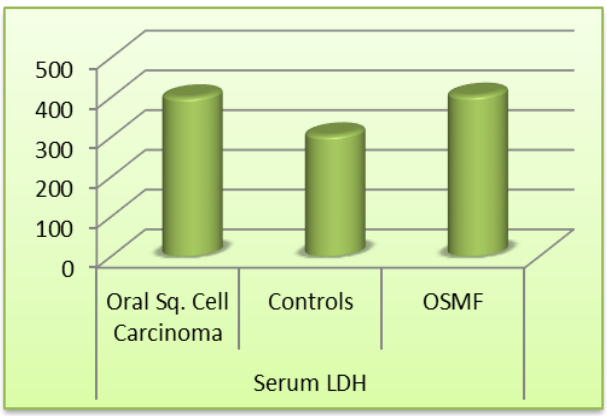
The data obtained were subjected to statistical analysis using the SPSS software version .

RESUTS:

Table-1: Mean & Sd For Serum & Saliva Ldh Levels

Table-1: MEAN & SD FOR SERUM & SALIVA LDH LEVELS						
		N	Mean	Std. Deviation	Minimum	Maximum
Serum LDH	Oral Sq. Cell Carcinoma	25	398.9577	62.23859	202.00	508.00
	Controls	25	303.3222	85.15201	177.40	515.00
	OSMF	25	403.1696	99.38947	281.00	698.00
	Total	75	366.2566	94.42935	177.40	698.00
Saliva LDH	Oral Sq. Cell Carcinoma	25	331.1538	118.15196	203.00	653.40
	Controls	25	159.2504	57.56644	60.00	235.00
	OSMF	25	299.8652	193.97478	44.00	843.00
	Total	75	260.6139	150.78270	44.00	843.00

BarGraph 1 of serum LDH mean:



BarGraph 2 of saliva LDH mean:

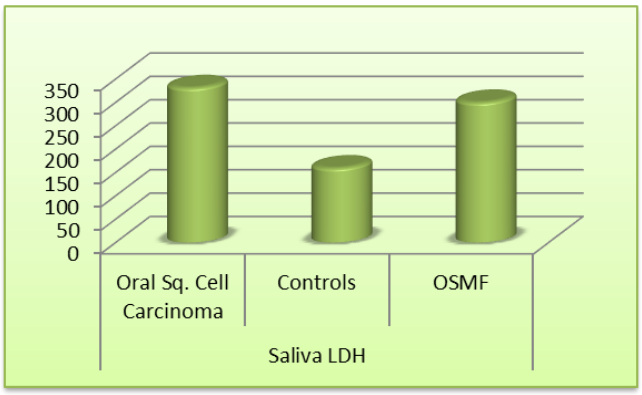


Table -2: Inter group comparison for statistical significance by ANOVA

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	Groups	Groups	Sig.
SerumLDH	Oral Sq. Cell Carcinoma	Controls	.001
		OSMF	.860
	Controls		
		OSMF	.001
SalivaLDH	Oral Sq. Cell Carcinoma	Controls	.001
		OSMF	.409
	Controls		
		OSMF	.001

Table-3: Intra & Inter group comparison by ANOVA for statistical significance

Table-3: Intra & Inter group comparison by ANOVA for statistical significance			
		F	Sig.
Serum LDH	Between Groups	12.059	.001
	Within Groups		
	Total		
Saliva LDH	Between Groups	12.781	.001
	Within Groups		
	Total		

According to Table 2 & 3 : The mean difference is significant at the 0.05 level

DISCUSSION:

OSCC is one of the most common head and neck malignancy with a worldwide incidence of over 300,000 new cases emerging per year and accounting for 2-4% of all new cancers.[18] There is an imperative need for developing new diagnostic tools that would improve early detection. The identification of molecular markers in body fluids that would predict the development of cancer in its earliest stage or in precancerous stage would constitute such a tool.

Tobacco usage either in smoking or nonsmoking form and alcohol consumption are considered to be most important causes of oral precancer or cancer. Nicotine affects a variety of cellular processes ranging from induction of gene expression to secretion of hormones and modulation of enzymatic activity. Cigarette smoking, which is one of the potent oxidant and radiation exposures that may exacerbate its effect, may induce functional and chemical change in living systems. Increased LDH levels are usually found due to cell death and/or leakage from the cells.[14]

The study was conducted with the aim of finding a predictive marker for potential malignancy, which can be applied to the rural Indian population. In this study lesions were diagnosed purely based on clinical examination, without usage of a biopsy. The principle of the current study was to find an alternative mass screening, non-invasive, cost-effective

procedure of a predictive marker for indicating the status of the lesion as potential malignancy ,malignancy or no malignancy .

Studies on analysis of salivary LDH either total or its isoenzyme levels in Oral submucous fibrosis and OSCC patients have not been carried out extensively. In our study though with a small sample size ,we found a statistically significant increase in both serum & saliva LDH in OSMF & OSCC patients as compared to controls.

This finding is in agreement with the studies done by Shpitzer *et al*.(19) who found total salivary LDH (88%, *P* = 0.002) level to be high in oral cancer patients. Our results are comparable with the study done by Shetty *et al*.[20] who have reported consistently higher salivary LDH levels in oral precancer and cancer and mean salivary LDH levels to be higher in males in comparison to females in all three study groups of leukoplakia, cancer and healthy controls.

The salivary LDH levels in OSMF cases (Group 2) were significantly higher than healthy subjects (Group 1) (Bargraph 2). The comparison of salivary LDH levels between OSMF cases and healthy subjects was statistically significant (P<0.001) (Table-2 &3). On comparing the serum LDH levels in OSMF cases (Group 2) and healthy controls (Group 1), results of the present study show that the LDH levels in serum of OSMF cases were greater than healthy control (Bargraph 1). This comparison was also statistically significant (P<0.001) (Table-2 &3)

Mean salivary LDH levels of OSCC subjects were significantly higher than OSMF group subjects. These findings are supported by the studies conducted by other investigators also. Joshi *et al*. [21] found increased serum and salivary LDH levels in all cases in Oral Leukoplakia(OL) and OSCC groups in comparison with the control group, but the increase in LDH levels was less in saliva as compared to serum in group II OL patients. The increase in LDH levels was consistent in saliva and serum of OSCC patients in his study.

Anuradha *et al*. (1998) in their study found that the activity of LDH in the serum of OSMF patients was significantly higher than the normal patients [22]. Kamath *et al*. (2013) in their study found that tissue breakdown releases LDH and therefore, LDH can be measured as a surrogate for tissue breakdown, e.g., hemolysis. Elevated levels were seen in OSMF patients indicating evidence of tissue breakdown.[23]

Data from the study support the initial hypothesis that LDH levels significantly increase in patients with potentially malignant lesions and, therefore, can act as a viable predictor, in the absence of other medical diseases and conditions, for potential malignancy. These patients should be followed up to find if, potentially malignant lesions eventually develop malignancy or not . Irrespective

of this study's findings, the potential benefits of LDH screening are enormous.

CONCLUSION:

The clinical diagnosis supplemented with LDH levels can gain diagnostic importance in near future. Moreover, the LDH levels can also be used as valuable aid in monitoring treatment outcomes in the OSMF & OSCC patients. The results of our study suggest that elevation of LDH above baseline is a specific marker for OSCC and OSMF.

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