RESULTS

4.1: EXPRESSION OF BIOMARKERS IN TISSUES OF ORAL CANCER PATIENTS

Biomarkers help in finding out the stage of the disease during prognostication and also assist in treatment monitoring. Evaluation expression of biomarkers in tissues is a better index of looking at the behaviour of cancerous cells as they signify and reflect the activity and local concentration *in vivo*. Lysates of cytosolic and nuclear fractions were prepared from tissues of oral cancer patients for assessing the biomarkers in the patients.

4.1.1 Expression of NFκB p65 in oral cancer:

The importance of transcription factor NFκB in promoting tumorigenesis has been well documented. It is known to be aberrant in many tumors with its potential implication in early as well as advanced stages of cancer. It modulates multitude of critical genes including biomolecules mediating several of events associated with multiple processes including acquisition of features such as promotion of cell survival and dysregulation of proliferation, angiogenesis, invasion and metastasis. Under normal conditions, NFκB being bound to inhibitory protein known as IκB is retained in the cytoplasm of cells. But when activated, NFκB is released from IκB inhibition and then translocated into the nucleus where it binds with the consensus sequence of target DNA. Therefore, to estimate the activation of NFκB p65, nuclear lysates were prepared from oral tissues and analysed by ELISA (quantitative assay) and western blot (to confirm Ab specificity and protein expression). The monoclonal antibody against human NFκB p65 for western hybridization studies was procured from Calbiochem. The commercial available ELISA kits were procured from Imgenex.

The protein levels of NFκB p65 was analyzed from nuclear extracts of malignant as well as adjacent normal tissues of oral cancer patients. NFκB-p65 standards were used to calculate the unknown levels of NFκB p65 in oral tissues and were run in every batch of the ELISA assay. **Figure-4.1.1** shows the standard curve plotted for NFκB p65. The representative pattern for expression of NFκB p65 and β-actin is shown in **Figure-4.1.2** estimated by

western hybridization. The bands obtained in the blots were scanned densitometrically using gel documentation system and results were represented as O.D/cu.mm^3.

Figure-4.1.1: Standard curve for NFκB p65

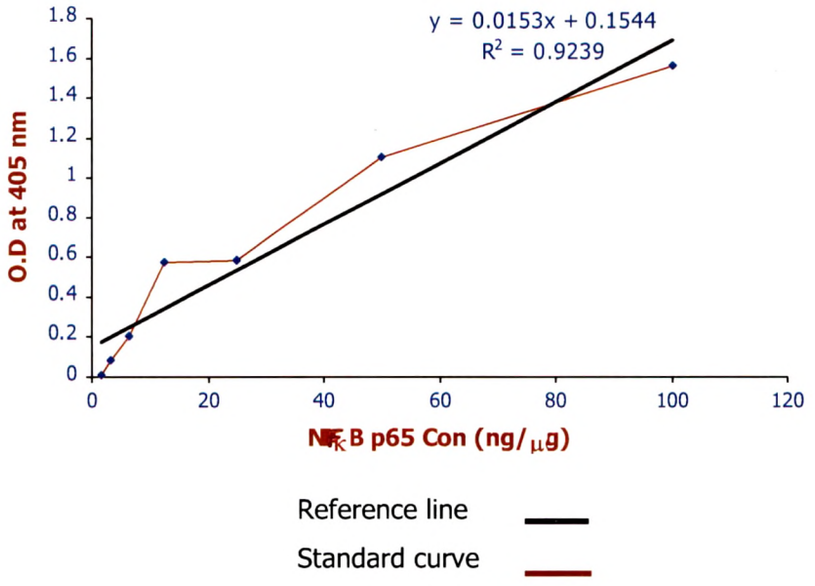
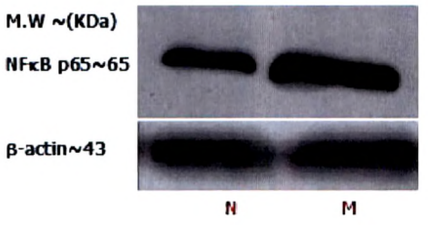


Figure-4.1.2: Representative pattern for NFκB p65 expression in tissues by western hybridization



N= Adjacent normal oral tissues **M**=Malignant oral tissues

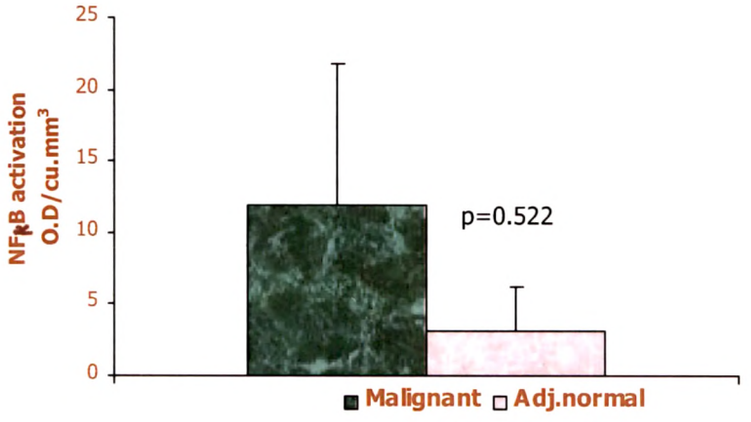
The differences in the means of biomarker between two groups of oral tissues were compared by student's *t*-test (unpaired and paired). The levels of mean NFκB p65 proteins were found to be higher in malignant as compared to adjacent normal tissues of oral cancer patients and their mean±S.E.M. is tabulated in **Table-4.1.1.**

Table-4.1.1: Comparison of mean values of NFκB p65 activation in oral cancer tissues

Biomarker	Oral Cancer Tissues mean±S.E.M.		Student's *t* -test
	Adj. normal	Malignant	'p' value
NFκB p65 (ng/μg) (ELISA)	0.7608±0.299	1.109±0.297	0.429[#] 0.235[$]
NFκB p65 (O.D /cu.mm^3) (Western hybridization)	3.129±3.129	11.974±9.724	0.408[#] 0.522[$]

[#]=Unpaired student's *t*-test
[$]= Paired student's *t*-test.

Figure-4.1.3: Expression of NFκB p65 protein in tissues by western hybridization



Moreover, the activation of NFκB p65 was also estimated using ELISA in the nuclear extracts of malignant and adjacent normal oral tissues of oral cancer patients. The mean±S.E.M. of NFκB p65 in nuclear extracts of adjacent normal tissues is 0.7608±0.2993 and the mean±S.E.M. of NFκB p65 in nuclear extracts of malignant tissues is 11.090+0.2974 as shown in **Table-4.1.1**. Malignant oral tissues showed higher activation as compared to their normal counterparts of these oral cancer patients (p=0.408).

Receiver's Operating Characteristic Curve analysis

Receiver's Operating Curve (ROC) is a more meaningful statistical analysis for discrimination between two groups under the study and also to analyze the diagnostic value of the markers. It simultaneously considers both the

specificity as well as sensitivity of the parameter. The table of ROC obtained for the test marker represents the area under the curve (AUC) as over all index for the diagnostic performance of the respective marker. if the AUC is below the reference green line, then it validates the parameter as diagnostic marker to differentiate between two groups under study. The area under the curve for the ROC analysis showing 95% CI and statistical significance of the test for NFκB p65 in oral cancer tissues is tabulated in **Table-4.1.2.** ROC curve for NFκB p65 is shown in **Figure-4.1.4** and it suggests that NFκB p65 had good discriminatory efficacy between adjacent normal and malignant tissues of oral cancer patients as documented by their area under the curve.

Figure-4.1.4: ROC curve for NFκB p65 in oral cancer patients

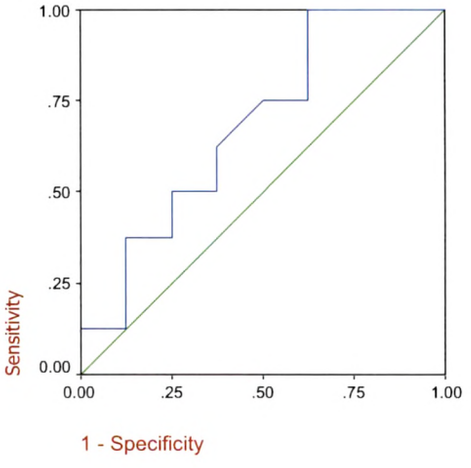


Table-4.1.2: Area under ROC curve for NFκB p65 in oral cancer patients

Biomarker	Area under the curve (AUC)	'p' value	95% CI	
			Lower bound	Upper bound
NFκB p65	0.618	0.227	0.410	0.949

The frequency of expression of NFκB p65 as present or absent in oral tissues is shown in **Table-4.1.3**. The activation of NFκB p65 was detected in 50% of the malignant tissues and in 27.8% of the adjacent normal tissues.

Table-4.1.3: Frequency of NFκB p65 expression in malignant and adjacent normal oral tissues

Oral cancer	NFκB p65 Activation	
	ABSENT	PRESENT
Malignant oral tissue	50%	50%
Adjacent normal oral tissues	72.23%	27.78%

Comparison of NFκB p65 activation with the clinico-pathological factors in oral cancer patients:

Oral cancer patients were assessed for NFκB p65 activation and associated with various clinical details of the patients as shown in **Table-4.1.4**. Clinical details like tobacco habit, tumor differentiation, tumor grade, lymph node status and stage of the disease were given with frequency of NFκB p65 activation.

Table-4.1.4: Status of NFκB p65 activation with clinico-pathological factors in oral cancer patients.

Parameter		NFκB p65 activation Positivity (/%)
Tobacco habit	WHT	87.5%
	NHT	12.5%
Tumor size	T1+T2	62.5%
	T3+ T4	37.5%
Differentiation	Well	37.5%
	Moderate	62.5%
Nuclear Grade	1	NIL
	2	87.5%
Nodal metastasis	Yes	37.5%
	No	50%
Stage	Early	25%
	Advanced	75%

NFκB p65 activation was present in 87.5% of patients with the habit of tobacco, 37.5% patients having tumor size 3 or 4. The activation was also observed in 37.5% and 62.5% of patients with well and moderately differentiated tumors respectively, 87.5 % of the patients with nuclear grade II, 37.5% of the patients with lymph node metastasis, and 75% of the patients with advanced disease.

Further, to determine the association of NFκB p65 activation with various clinico-pathological parameters like age, sex, tobacco habit, tumor-differentiation, nuclear grade, lymphatic response, tumor infilteration, lymph-

node metastasis and stage of the disease, multivariate analysis was carried out (**Table-4.1.5**). It was observed that NFκB p65 activation was closely associated with tumor infilteration (p=0.087) and stage of the disease (p=0.057) in the malignant tissues. However, remaining clinicopathological parameters did not reveal any significant association with NFκB p65. Thus, NFκB p65 activation might serve as an independent variable in oral carcinogenesis.

Table-4.1.5: Association of NFκB p65 activation with clinico-pathological parameters in oral cancer patients by Multivariate analysis.

NFκB p65 activation	**Adjacent normal tissues**		**Malignant tissues**	
Clinico-pathological parameters	**F value**	**'p' Value**	**F value**	**'p' value**
Age	0.811	0.402	0.821	0.400
Sex	1.735	0.230	2.202	0.188
Habit	0.439	0.532	0.046	0.836
Tumor Differentiation	2.404	0.172	1.657	0.245
Lymphatic response	1.567	0.279	3.269	0.145
Tumor infilteration	1.626	0.258	4.503	0.087
Lymph-Node metastasis	0.608	0.471	0.567	0.485
Tumor size	0.220	0.655	0.893	0.381
Stage of the disease	0.257	0.445	2.309	0.195
Early/advanced stage	2.267	0.183	5.540	0.057

Correlation of NFκB p65 activation with other parameters studied:

Bivariate correlation like Pearsons correlation was calculated for comparing correlation of NFκB p65 activation with other biomarkers studied in tissue and blood samples (Table-4.1.6). A significant positive correlation was observed between expression of NFκB p65 in malignant and adjacent normal tissues of oral cancer patients (p=0.001). A significant positive correlation was also observed between NFκB p65 activation and Bcl-2/Bax ratio (p=<0.0001) in malignant tissues. NFκB p65 activation in malignant tissues was found to correlate positively with expression of Bax, latent and active forms of MMP-2 but negatively correlated with latent and active MMP-9 in malignant tissues. Comparing NFκB p65 activation with circulating factors revealed that a

positive correlation between serum IL-8 and glycoprotein constituents and negatively correlated with serum p53 autoantibodies in oral cancer patients.

Table-4.1.6: Correlation of NFκB p65 activation in malignant tissues with other factors in oral cancer patients

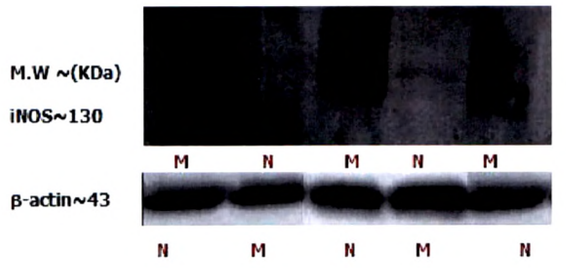
Factors	Pearsons correlation	Significance Two-tailed
Adjacent normal tissues		
NFκB p65	0.924	**0.001**
Malignant tissues		
Bax	0.415	0.488
Bcl-2/Bax	0.997	**>0.0001**
Latent MMP-2 Active MMP-2	0.147 0.021	0.728 0.961
Latent MMP-9 Active MMP-9	-0.131 -0.521	0.757 0.185
Serum p53 autoantibodies	-0.173	0.711
Serum IL-8	0.120	0.821
Serum TSA	0.601	0.207
Serum Mucoid Protein	0.281	0.510
Serum Hexoses	0.263	0.615

4.1.2 Expression of iNOS in oral cancer patients

Inflammation, angiogenesis as well as other aspects of carcinogenesis is modulated by expression of nitric oxide produced by inducible form of nitric oxide synthase (iNOS) enzyme in tumor and stromal cells, which also affects the response of patients to treatment. There have been studies of tumor origin including head and neck squamous cell carcinoma (HNSCC) indicating significantly higher expression of iNOS and higher NO levels in tumor tissues compared to their counter normal tissues and this event appears to be crucial for invasion and metastasis [Emma et al., 2005; Park et al., 2003]. The expression of iNOS protein was estimated in malignant as well as adjacent normal tissues of oral cancer patients by western blot. The monoclonal antibody against human iNOS recognizes the band at 130 KDa and was procured from R & D Systems. **Figure-4.1.5** shows the representative blot for expression of iNOS in malignant as well as adjacent normal tissues of oral cancer patients. The blots were scanned densitometrically and expressed as O.D/cu.mm^3 by using gel documentation system. iNOS expression was

observed in both adjacent normal and malignant tissues of oral cancer patients. The mean±S.E.M. of iNOS was found to be higher malignant (p=0.581) as compared to adjacent normal tissues of oral cancer patients (**Table-4.1.7** and **Figure-4.1.6**).

Figure-4.1.5: Representative pattern for iNOS expression in tissues



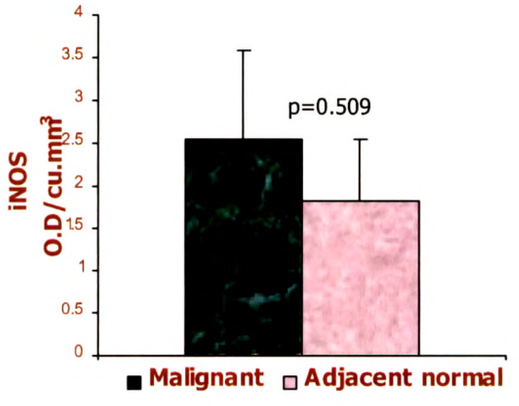
N= Adjacent normal oral tissues **M**=Malignant oral tissues

Table-4.1.7: Comparison of mean values of iNOS in oral cancer tissues

Biomarker	Oral Cancer Tissues Mean±S.E.M		Student's *t* -test
	Adj. normal	Malignant	"p" value
iNOS	1.8189±0.7194	2.5365±1.0408	0.581[#] 0.509[$]

[#]=Unpaired Student's *t* -test
[$]= Paired Student's *t* -test

Figure-4.1.6: iNOS expression in malignant and adjacent normal oral tissues



The frequency of expression of iNOS protein as present or absent in oral tissues is shown in **Table-4.1.8**. 87.5% of malignant tissues showed the presence, while 81.25% of adjacent normal tissues showed the presence of iNOS protein.

Table-4.1.8: Frequency of iNOS expression in malignant and adjacent normal oral tissues

Oral cancer	iNOS expression	
	ABSENT	PRESENT
Malignant oral tissue	12.57%	87.5%
Adjacent normal oral tissues	18.75%	81.25%

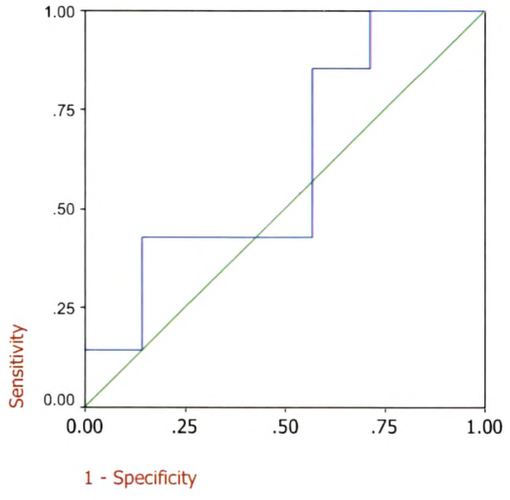
Receiver's Operating Characteristic Curve analysis

ROC was also plotted to evaluate the discriminatory efficacy of iNOS and it revealed that iNOS had good efficacy to discriminate between the malignant and adjacent normal tissues in oral cancer patients as documented by there area under the curve (**Figure-4.1.7**). The area under the curve for the ROC analysis showing 95% CI and statistical significance of the test for iNOS in oral cancer tissues is tabulated in **Table-4.1.9**

Table-4.1.9: Area under ROC curve for iNOS in oral cancer patients

Biomarker	Area under the curve	'p' value	95% CI	
			Lower bound	Upper bound
iNOS	0.612	0.482	0.301	0.923

Figure-4.1.7: ROC curve for iNOS expression in oral cancer patients



Comparison of iNOS expression with the clinico-pathological parameters in oral cancer patients:

The frequency of iNOS expression was compared by multivariate analysis with tobacco habits as well as with the various clinical details like tobacco habit, sex, age, tumor differentiation, tumor grade, lymph node status and stage of the disease to know their association with iNOS expression in adjacent normal and malignant tissues of the oral cancer patients and the results are shown in **Table-4.1.10**. iNOS expression in adjacent normal tissues was found to be significantly higher (p=0.004) in male oral cancer patients and non-tobacco users as compared to female cancer patients and tobacco users. The iNOS expression in malignant tissues was found to be significantly higher (p=0.01) in well-differentiated tissues as compared to moderately differentiated tissues of oral cancer patients.

Table-4.1.10: Association of iNOS expression with clinico-pathological parameters in oral cancer patients by Multivariate analysis.

Clinico-pathological parameters	iNOS expression			
	Adjacent normal tissues		Malignant tissues	
	F value	'p' value	F value	'p' Value
Age	0.138	0.725	0.743	0.428
Sex	25.862	**0.004**	0.191	0.680
Habit	25.862	**0.004**	0.191	0.680
Tumor Differentiation	0.00	0.987	16.429	**0.01**
Lymphatic response	1.609	0.332	2.488	0.255
Tumor infilteration	1.008	0.361	3.022	0.143
Lymph-Node metastasis	0.495	0.332	1.291	0.319
Tumor size	0.741	0.438	0.720	0.444
Stage of the disease	0.377	0.573	2.043	0.226
Early/advanced stage	0.377	0.573	2.043	0.226

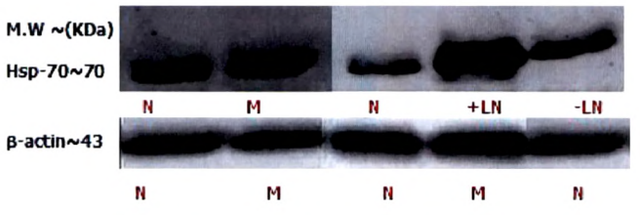
However, the iNOS expression in malignant tissues was higher in patients having lymphatic response (p=0.255) and tumor infilteration (p=0.143) as compared to those who did not have. However, no association was observed

with the other clinicopathological parameters in the malignant as well as adjacent normal tissues.

4.1.3 Expression of Hsp-70 in oral cancer patients

Hsp-70 is one of the most abundant chaperone expressed in mammalian tissues. It is known to affect treatment response by modulating the apoptotic functions in tumor cells. It not only modulates by interacting with house keeping genes like p53 but also affecting key apoptotic proteins like Bcl-2 and Bax in cancerous cells. The Hsp-70 proteins are estimated by western blot technique using monoclonal antibodies against human Hsp-70 recognizing the band of 70 KDa procured from R & D Systems. **Figure-4.1.8** shows the representative blot obtained for expression of Hsp-70 in malignant, adjacent normal, lymph-node positive and negative tissues of oral cancer patients. The blots were scanned densitometrically and expressed as O.D/cu.mm^3 by using gel documentation system. Expression of Hsp-70 protein was observed in malignant, adjacent normal and lymph-node tissues of the patients with oral squamous cell carcinoma (OSCC).

Figure-4.1.8: Representative pattern for Hsp-70 expression in OSCC tissues



N= Adjacent normal oral tissues **M**=Malignant oral tissues
+LN= Positive Lymph-node ; **-LN**= negative Lymph-node

Paired and unpaired student's *t*-test was carried out to compare the Hsp-70 expression between malignant and adjacent normal tissues (**Table-4.1.11**). The mean levels of Hsp-70 proteins were found to be higher (p=0.269) in malignant as compared to adjacent normal tissues of oral cancer patients (**Figure-4.1.9**).

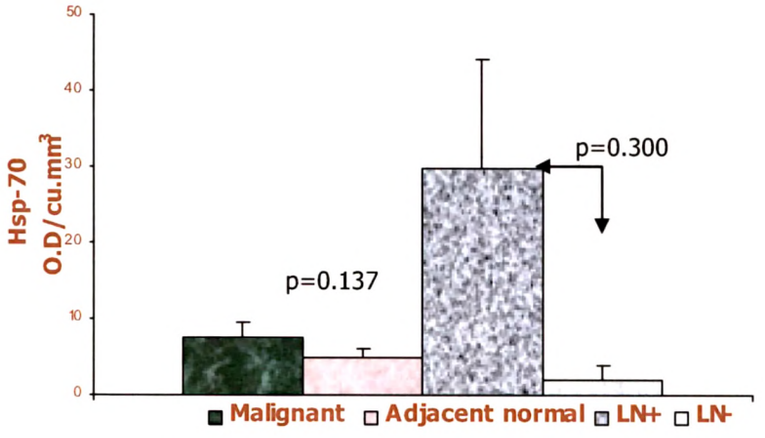
Table-4.1.11: Comparison of mean values of Hsp-70 in oral cancer tissues

Biomarker	Oral Cancer Tissues Mean±S.E.M		Student's *t*- test
	Adj. normal	**Malignant**	**'p' value**
Hsp-70	4.8087±1.3464	7.5519±1.9413	0.265 [#] 0.137[$]
	Lymph-node negative	**Lymph-node positive**	
Hsp-70	1.9490±1.9490	29.6755±14.3675	0.339[#] 0.300[$]

[#]=Unpaired Student's *t* –test [$]= Paired Student's *t* -test

The frequency of expression of Hsp-70 as present or absent in oral tissues is shown in **Table-4.1.12**. 90% malignant tissues showed the presence of Hsp-70 protein, while 80% of adjacent normal tissues showed the presence of Hsp-70.

Figure-4.1.9: Hsp-70 expression in oral SCC tissues



(+LN=Lymph-node positive; -LN =Lymph-node negative)

Table-4.1.12: Frequency of Hsp-70 expression in malignant and adjacent normal oral tissues

Oral cancer	Hsp-70 expression	
	ABSENT	**PRESENT**
Malignant oral tissue	10%	90%
Adjacent normal oral tissues	20%	80%

Receiver's Operating Characteristic Curve analysis

Receiver's Operating Curve (ROC) was also plotted to evaluate the discriminatory efficacy of Hsp-70 and the analysis revealed that Hsp-70 had

good efficacy to discriminate between malignant and adjacent normal tissues in oral cancer patients (**Figure-4.1.10)**. The area under the curve for the ROC analysis showing 95% CI and statistical significance of the test for Hsp-70 in oral cancer tissues is tabulated in **Table-4.1.13**

Figure-4.1.10: ROC curve for Hsp-70 expression in oral cancer patients

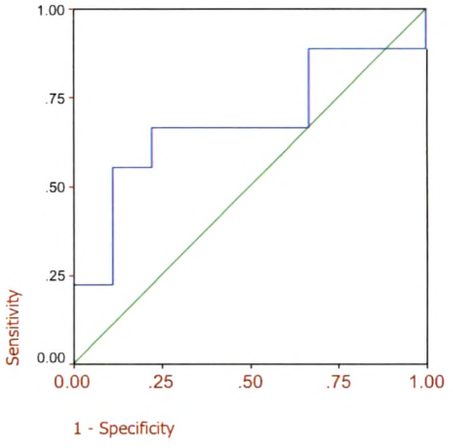


Table-4.1.13: Area under ROC curve for Hsp-70 expression in oral cancer patients

Biomarker	Area under the curve	'p' value	95% CI	
			Lower bound	Upper bound
Hsp-70	0.679	0.200	0.415	0.943

Comparison of Hsp-70 expression with the clinico-pathological parameters in oral cancer patients:

The mean levels of Hsp-70 expression were compared with the clinical details as well as with the tobacco habits of the oral cancer patients. Details like tobacco habit, sex, age, tumor differentiation, tumor grade, lymph node status and stage of the disease were associated with Hsp-70 expression in adjacent normal and malignant tissues by unpaired student's *t*-test analysis. Hsp-70 expression in adjacent normal tissues was found to be significantly higher (p=0.036) in advanced stage as compared to early stage of the disease of oral cancer patients.

Table-4.1.14: Association of Hsp-70 expression with clinico-pathological parameters in oral cancer patients by Multivariate analysis

Hsp-70 expression	Adjacent normal tissues		Malignant tissues	
Clinico-pathological parameters	F value	'p' value	F value	'p' value
Age	0.36	0.856	2.663	0.147
Sex	0.231	0.646	2.065	0.194
Habit	0.231	0.646	2.065	0.194
Tumor Differentiation	2.309	0.203	0.762	0.416
Lymphatic response	0.008	0.936	0.598	0.496
Tumor infilteration	3.255	0.121	0.121	0.740
Lymph-Node metastasis	0.001	0.971	2.901	0.139
Tumor size	0.572	0.483	0.329	0.591
Stage of the disease	8.062	**0.036**	0.140	0.760
Early/advanced stage	8.062	**0.036**	0.140	0.760

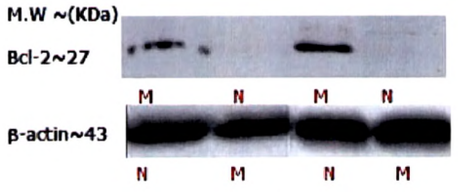
Further, multivariate analysis was also carried out to determine the association of Hsp-70 expression in paired oral tissues with various clinico-pathological parameters (**Table-4.1.14**). Expression of Hsp-70 in adjacent normal tissues was found to be significantly associated with stage of the disease (p=0.036) in oral cancer patients. nonetheless, no significant association was observed between Hsp-70 expression in adjacent normal as well as malignant tissues and other clinicopathological parameters.

4.1.4 Expression of Bcl-2 and Bax in oral cancer patients

Bcl-2 and Bax play critical role in apoptosis. Bax has been established as a tumor suppressor, because Bax inactivation leads to rapid tumor growth by decreasing apoptosis of tumor cells. The monoclonal antibodies against human Bcl-2 and Bax were procured from R & D Systems. The levels of Bcl-2 (26 kDa) and Bax (21 kDa) were estimated using western blot analysis. **Figures 4.1.11 and 4.1.12** show the representative blots obtained for expression of Bcl-2 and Bax respectively in malignant and adjacent normal tissues of oral cancer patients. The blots were scanned densitometrically and expressed as O.D/cu.mm^3 by using gel documentation system. The expression of Bcl-2 was more prominent in malignant tissues as compared to adjacent

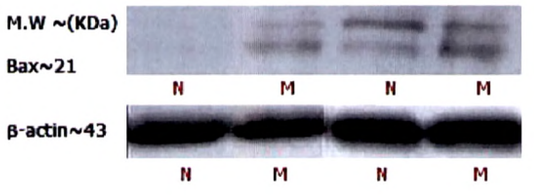
normal tissues, while the expression of Bax protein was observed in both malignant and adjacent normal tissues of OSCC patients.

Figure-4.1.11: Representative pattern for Bcl-2 expression in tissues



N= Adjacent normal oral tissues **M**=Malignant oral tissues

Figure-4.1.12: Representative pattern for Bax expression in tissues



N= Adjacent normal oral tissues **M**=Malignant oral tissues

Student's *t*-test was carried out to compare the Bcl-2 and Bax expression between malignant and adjacent normal tissues (**Table-4.1.15**).

Table-4.1.15: Comparison of mean values of apoptotic proteins in oral SCC tissues

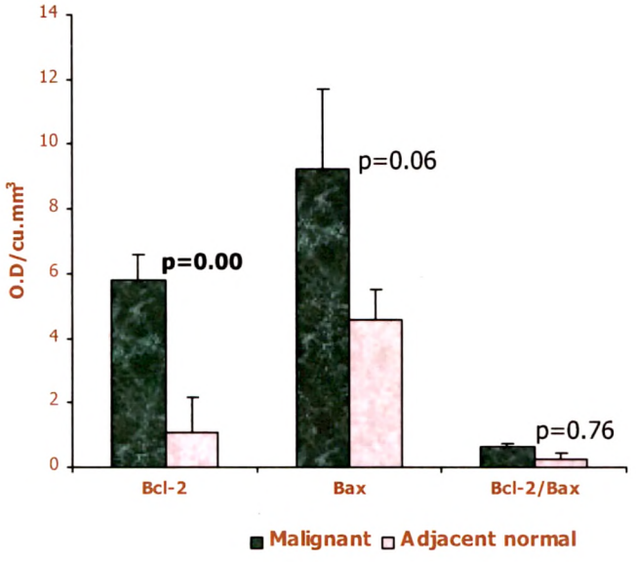
Biomarkers	**Oral Cancer Tissues Mean±S.E.M**		**Student's *t*-test**
	Adj. normal	**Malignant**	**'p' value**
Bcl-2	1.0808±1.0808	5.8150±0.7773	**0.004**[#] **0.018**[$]
Bax	4.5523±0. 9385	9.2364±2.4533	0.101[#] **0.06** [$]
Bcl-2/Bax	0.2065±0.2065	0.2591±0.115	0.827[#] 0.765 [$]

[#]=Unpaired Student's *t* –test [$]= Paired Student's *t* -test

The levels of oncoprotein Bcl-2 was found to be significantly higher (p=0.004) in malignant as compared to adjacent normal tissues of oral cancer patients (**Figure-4.1.13).** Nevertheless no significant change was

found in the Bax expression in malignant tissue as compared to adjacent normal tissue. But paired analysis revealed that bax protein was higher in malignant tissues (p=0.06) as compared to its normal counter part in oral cancer patients.

Figure-4.1.13: Expression of apoptotic proteins in malignant and adjacent normal oral tissues



The band intensities for the levels of Bcl-2 protein were found to be significantly higher (p=0.004) in malignant as compared to adjacent normal tissues of oral cancer patients. The mean levels of band intensities for Bax protein was found to be higher (p=0.06) in malignant as compared to adjacent normal tissues of oral cancer patients (**Figure-4.1.13)**. The ratio of Bcl-2/ Bax was also calculated as they serve as better index. The ratio of Bcl-2/ Bax was higher (p=0.827) in malignant and adjacent normal tissues as compared to their counterparts. The frequency of expression of Bcl-2 and Bax proteins in oral cancer patients is shown in **Table-4.1.16.** 71.4% of malignant and 14.3% of adjacent normal tissues showed the presence of Bcl-2 protein, while 68.8 % of malignant and adjacent normal tissues showed the presence of Bax protein as assessed in paired oral tissues.

Table-4.1.16: Frequency of Bcl-2 and Bax expression in malignant and adjacent normal oral tissues

	Bcl-2		Bax	
Oral tissues	**ABSENT**	**PRESENT**	**ABSENT**	**PRESENT**
Malignant tissue (M)	28.57%	71.43%	41.18%	68.82%
Adjacent normal (N)	85.71%	14.29%	41.18%	68.82%

Receiver's Operating Characteristic Curve analysis

Receiver's Operating Curve (ROC) was also plotted (**Figure-4.1.14)** to evaluate the discriminatory efficacy of Bcl-2 and Bax between adjacent normal and malignant tissues of oral cancer patients. ROC curve analysis suggested that bcl-2, Bax and bcl-2/bax ratio had good efficacy to discriminate between the malignant and adjacent normal tissues of oral cancer patients as shown in **table-4.1.17**. The area under the curve for the ROC analysis showing 95% CI and statistical significance of the test for apoptotic proteins and bcl-2/bax ratio in oral cancer tissues is tabulated in **table-4.1.17.**

Figure-4.1.14: ROC curve for Bcl-2 in oral cancer patients

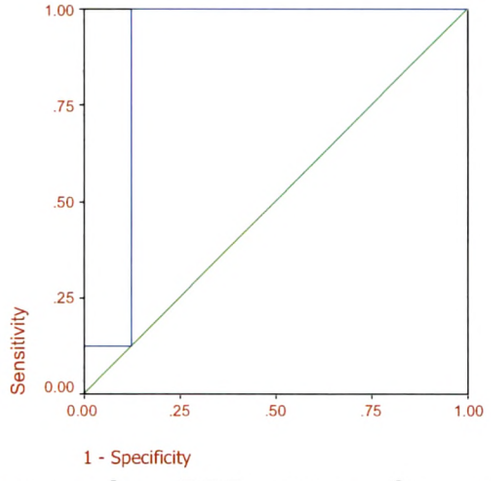


Table-4.1.17: Area under ROC curve for apoptotic proteins expression in oral cancer patients

Biomarker	Area under the curve	'p'value	95% CI	
			Lower bound	*Upper bound*
Bcl-2	0.891	**0.009**	0.686	1.095
Bax	0.640	0.290	0.381	0.899
Bcl-2/bax	0.630	0.326	0.376	0.884

Comparison of apoptotic proteins expression with the clinico-pathological parameters in oral cancer patients:

The mean levels of both Bcl-2 and Bax were associated with the clinical details of the oral cancer patients. Details like tumor differentiation, tumor grade, lymph node status and stage of the disease were associated with Bcl-2 and Bax expression in malignant tissues by student's *t*-test analysis. The expression of Bcl-2 in malignant tissues was found to be significantly higher (p=0.014) in oral cancer patients with larger tumor size (T3+T4) as compared to lower tumor size (T1+T2) as shown in **Table-4.1.18.**

Table-4.1.18: Unpaired Student's *t*-test analysis of Bcl-2, Bax and Bcl-2/Bax ratio in malignant tissues with tumor size of oral cancer patients.

Biomarker	T1+T2 Mean±S.E.M	T3+T4 Mean±S.E.M	'p' value
Bcl-2	4.5173±0.678	7.3755±0.010	**0.014**
Bax	5.5522±1.666	14.7628±4.607	0.137
Bcl-2/Bax	0.3766±0.173	0.08271±0.082	0.170

Further, multivariate analysis was carried out to determine the association of Bcl-2 and Bax expression with various clinico-pathological parameters (**Table-4.1.19**). Nonetheless, no significant association was observed for expression of apoptotic proteins (Bcl-2 and Bax) with clinicopathological parameters in the malignant as well as adjacent normal tissues of oral cancer patients.

Table-4.1.19: Association of Bcl-2 and Bax expression in malignant tissues with clinico-pathological parameters in oral cancer patients by Multivariate analysis.

Clinico-pathological parameters	Bcl-2 Malignant tissues		Bax Malignant tissues	
	F value	'p' value	F value	'p' value
Age	0.000	0.988	0.243	0.671
Tumor Differentiation	0.011	0.927	0.279	0.650
Nuclear grade	0.256	0.702	0.005	0.954
Tumor infilteration	0.457	0.622	0.357	0.657
Lymph-Node metastasis	0.770	0.473	0.647	0.506
Tumor size	1.191	0.389	8.354	0.102
Stage of the disease	0.529	0.697	0.187	0.853
Early/advanced stage	0.011	0.927	0.279	0.650

4.1.6 Tissue Gelatinases in oral cancer

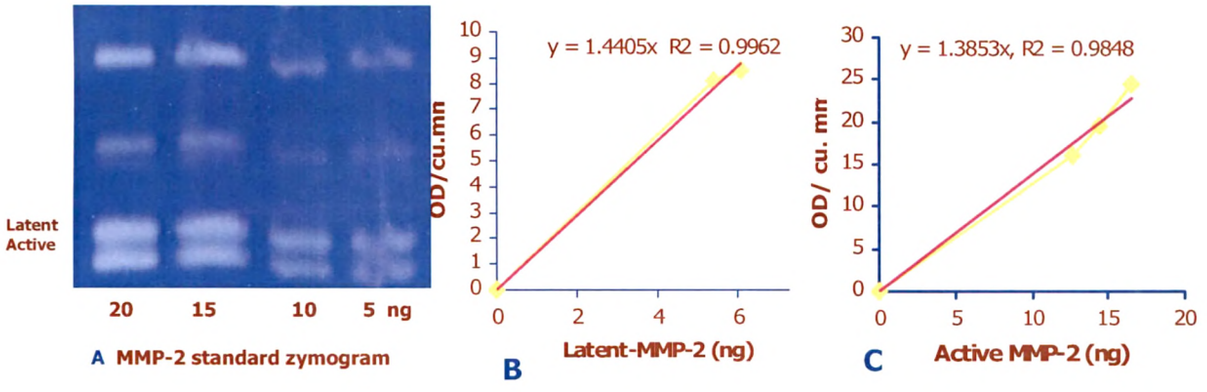
Tissue gelatinases both MMP-2 and MMP-9 were assessed by Gelatin zymography (substrate-zymography), a sensitive and specific functional assay discriminating both latent and active forms of gelatinases by gel analysis. All malignant (M), adjacent normal (N) and lymph-node (LN) positive and negative tissues were homogenized to yield tissue cytosolic preparations which were assessed for gelatinolytic activity by subjecting the lysates of oral tissue samples to gelatin SDS-PAGE zymography. 50 µg of sample was loaded onto each lane and concentrations of both the forms of MMP-2 and MMP-9 were calculated as ng/50 µg of protein using linear equation derived from standard zymograms. The resultant gels were densitometrically analysed using gel documentation system. MMPs are generally secreted as zymogens i.e., latent MMP-2 and latent MMP-9 *in vivo.* But when latent forms are renatured, they convert in to their active forms. The active forms of MMPs then degrade the gelatin impregnated in the SDS-PAGE gel and produce clear zone of white bands against the dark background of gels revealing enzymatic activity of MMPs as shown in the representative pattern (**Figures-4.1.16 and 4.1.20**). Both latent and active gelatinases constitute total MMP, which can be calculated for representing the total levels of these gelatinases present in the tissues of cancer patients reflecting the aggressive nature of cancer. Activation ratio was also calculated as concentration of active forms/concentration of total activity, as activation is prerequisite for invasive behaviour of cancer dissemination. The present study evaluated the prognostic significance of these gelatinases in patients with oral SCC.

Expression of Tissue Gelatinase-A (MMP-2) in oral cancer

Gelatinase-A or MMP-2 is implicated with aggressive behaviour of many solid cancers [Wood house et al., 1997]. Both the latent as well as active forms of MMP-2 were analysed by gelatin zymography. Concentration of MMP-2 was expressed as ng/50µg protein using latent and active human MMP-2 standards (Calbiochem). The representative zymogram and standard graph for latent and active MMP-2 are shown in **Figure 4.1.15**. The standards (0-

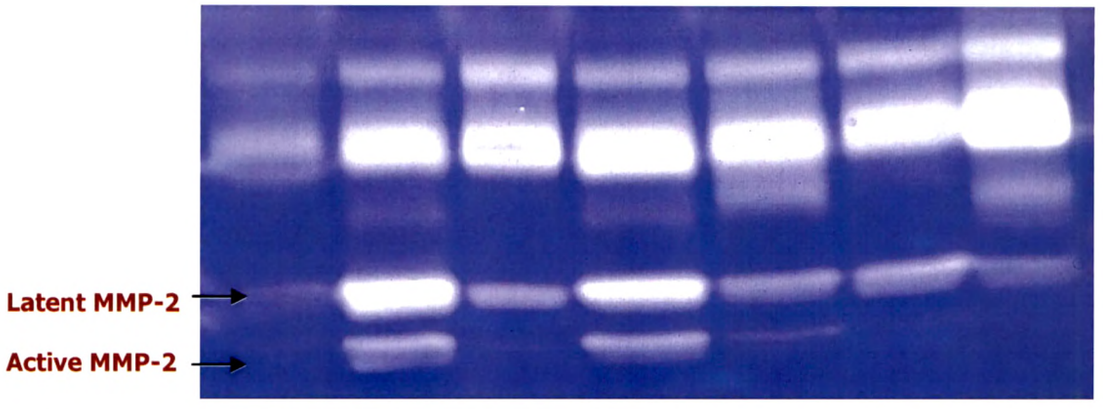
20ng) of latent and active forms of MMP-2 were subjected to gelatin zymography and the position of both forms are shown in the zymogram (**Figure 4.1.15 A**). The gel was densitometrically analysed and a standard graph was plotted for concentration of standard against the optical density per unit area and are used for analysis of unknown concentration of MMP-2. It yielded a linear correlation between standard concentration and activity of MMP-2 as plotted in graph. **Figure 4.1.15 B** and **Figure 4.1.15 C** show the linear standard graphs for latent MMP-2 and active MMP-2 respectively.

Figure-4.1.15: Representative gelatin zymogram and standard curves for gelatinase-A [MMP-2]: (A; Zymogram, B; latent, C; active) standards



Representative zymogram for MMP-2 expression in cytosolic preparation of oral SCC tissue samples of cancer patients is illustrated in **Figure-4.1.16**. Both latent as well as active MMP-2 are expressed in adjacent normal, malignant and lymph-node tissues as evident from the **Figure-4.1.16** showing their respective position as clear zones exhibiting their gelatinolytic activity. Interestingly the expression of MMP-2 activities is more prominent in malignant tissues compared to their counter parts. In addition to the above forms, total and activation ratio was also calculated for MMP-2 in these tissues, which is compared between adjacent normal and malignant tissues of the patients. Total MMP-2 was expressed as sum of latent and active forms of MMP-2 while activation ratio for MMP-2 was expressed as active MMP-2 divided by total MMP-2.

Figure-4.1.16: Representative pattern for gelatinase-A (MMP-2) in oral SCC tissues



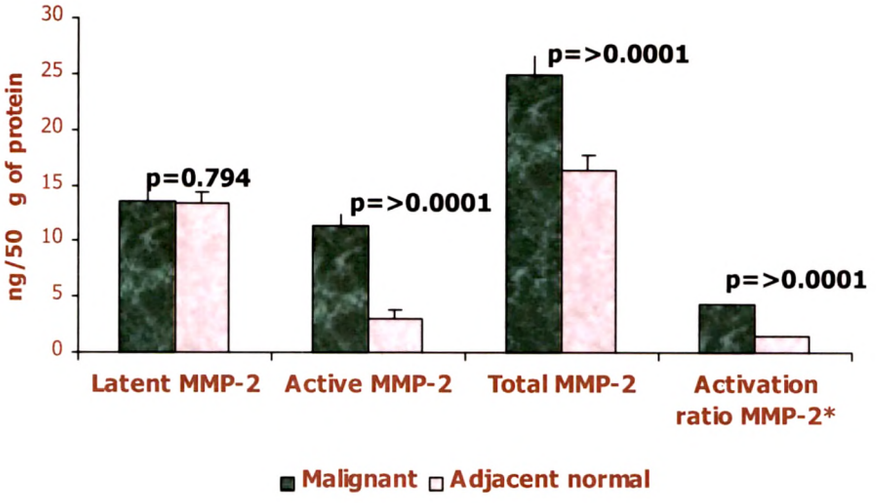
N=Adjacent normal oral SCC tissue **M**=malignant oral SCC tissues

+**LN**= positive lymph-node oral SCC tissue

Comparison of latent, active, total and activation ratio of MMP-2 in oral cancer tissues.

MMP-2 is expressed as mean± S.E.M in adjacent normal and malignant tissues of oral cancer patients and student's *t*-tests (paired and unpaired) were performed to compare the levels of different forms of MMP-2 and the results are tabulated in **Table-4.1.20**. It is clearly evident from the **Figure-4.1.17,** that the levels of all the forms were higher in malignant tissues as compared to adjacent normal tissues. The mean±S.E.M value for latent and active MMP-2 in adjacent normal tissues of oral cancer patients are 13.4472±0.9314 and 2.9983±0.8527 respectively. While the mean±S.E.M value for latent and active MMP-2 in malignant tissues of oral cancer patients are 13.6590±0.9677 and 11.3710±1.0304 respectively. Latent MMP-2 was higher in malignant tissues as compared to adjacent normal tissues (P=0.794). Active, total and activation ratio of MMP-2 was significantly elevated in malignant tissues as compared to adjacent normal tissues (p=>0.001, p=>0.001, and p=>0.001) respectively.

Figure-4.1:17 Comparison of latent, active, total and activation ratio of gelatinase-A (MMP-2) in oral SCC tissues



Activation Ratio MMP-2* =values divided by 10

Table-4.1.20: Comparison of mean values of different forms of gelatinase-A (MMP-2) in oral cancer tissues

Biomarkers	Oral Cancer Tissues Mean± S.E.M		Student's *t*-test
	Adj. normal	Malignant	'p' value
Latent MMP-2	13.4472±0.9314	13.6590±0.9677	0.875 # 0.794 $
Active MMP-2	2.9983±0.8527	11.3710±1.0304	**<0.001** # **<0.001** $
Total MMP-2	16.4455±1.3080	25.0300±1.6722	**<0.001** # **<0.001** $
Activation ratio of MMP-2	0.1446±0.0364	0.4406±0.0306	**<0.001** # **<0.0001** $

#=Unpaired student's *t*-test $= Paired student's *t*-test

Receiver's Operating Characteristic (ROC) curve analysis

Receiver's Operating Characteristic (ROC) curve analysis was plotted to evaluate the efficiency of MMP-2 to discriminate between malignant and adjacent normal tissues of oral cancer patients considering both the sensitivity and specificity of the biomarker under study (**Figure-4.1.18**). ROC curve analysis revealed that latent, active, total forms as well as activation ratio of MMP-2 have good efficacy to discriminate between adjacent normal and malignant tissues using zymography technique. The area under the curve for

ROC is tabulated in **Table-4.1.21** showing p value significance for all the forms of MMP-2.

Figure-4.1.18: ROC curve for gelatinase-A (MMP-2) in oral cancer patients

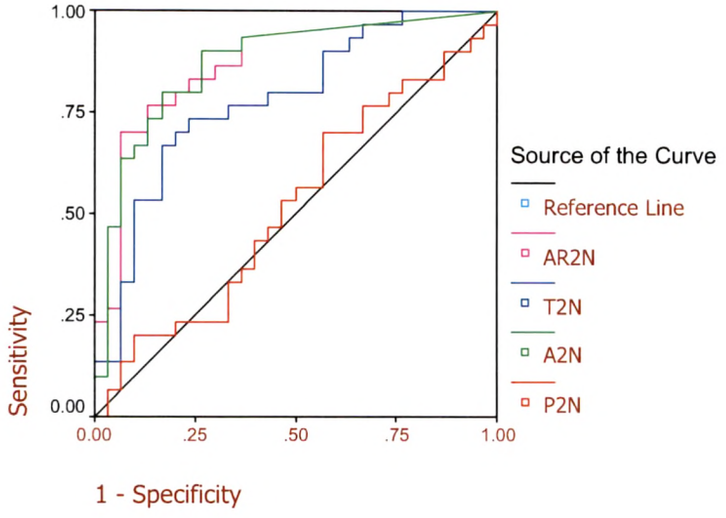


Table-4.1.21: Area under ROC curve for all forms of gelatinase-A (MMP-2) in oral cancer patients

Biomarkers	Area under the curve	'p' value	95% CI	
			Lower bound	Upper bound
Latent MMP-2	0.521	0.779	0.373	0.669
Active MMP-2	0.869	**0.001**	0.774	0.963
Total MMP-2	0.779	**0.001**	0.661	0.897
Activation ratio MMP-2	0.867	**0.001**	0.772	0.961

Comparison of gelatinase-A with the clinico-pathological parameters in oral cancer patients

Clinical details like age, sex, tobacco habit, tumor differentiation, nuclear grade, lymph node status, lymphatic response, tumor infilteration, tumor size, stage of the disease were associated with the expression of MMP-2 in adjacent normal and malignant tissues of oral cancer patients. The expression of all forms of MMP-2 in adjacent as well as malignant tissues was comparable with the clinical details of patients. The mean values for active MMP-2 were found to correlate significantly with lymphatic response and

tumor size as shown in **Table-4.1.22 and Figure-4.1.19**. Active MMP-2 expression in malignant tissues was significantly higher in oral cancer patients with lymphatic response as compared to those with no lymphatic response (p=0.043). While its expression in adjacent normal tissues was also found to be significantly higher in oral cancer patients with larger tumor size as compared to those with smaller tumor size (p=0.032).

Table-4.1.22: Association of gelatinase-A (MMP-2) with lymphatic response in oral cancer patients.

Biomarkers	**Lymphatic response** Mean± S.E.M ng/50 μg		**Unpaired Student's *t*- test**
Malignant tissues	**Yes**	**No**	**'p value**
Active MMP-2	13.3657±1.4798	7.8579±1.8442	**0.043**
	Tumor size		
Adjacent normal tissues	**T1+T2**	T3+T4	
Active MMP-2	0.9847±0.6667	4.6627±1.4512	**0.032**

Further, multivariate analysis was performed to evaluate possible correlation between MMP-2 expression in both the tissues with different clinico-pathological parameters including age, gender, site of tumour, stage of tumour, nodal involvement, histological grade and nuclear grade of tumour.

Figure-4.1.19: Comparison of gelatinase-A (MMP-2) in oral SCC with lymphatic response and larger tumor size.

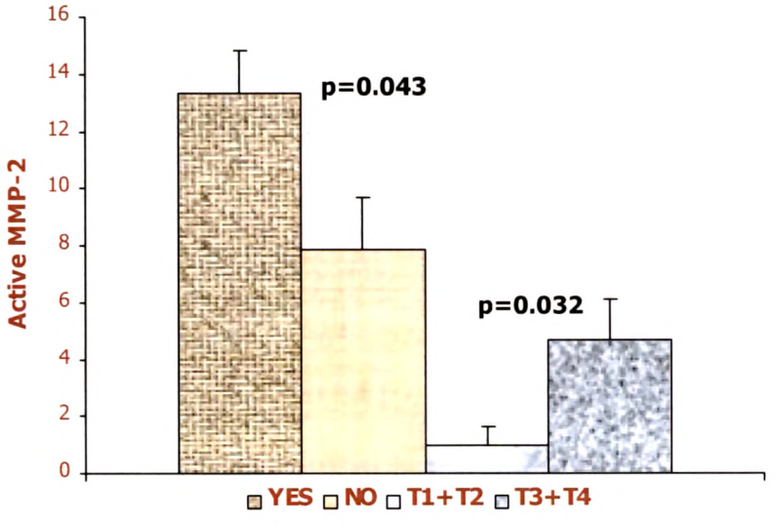


Table-4.23 shows correlation of latent, active, total forms and activation ratio of MMP-2 in malignant with clinicopathological factors in adjacent normal and malignant tissues by multivariate analysis. No significant correlation was observed between clinico-pathological factors and MMP-2 expression and activation.

Table-4.1.23: Association of gelatinase-A (MMP-2) with clinico-pathological parameters in oral cancer patients by Multivariate analysis.

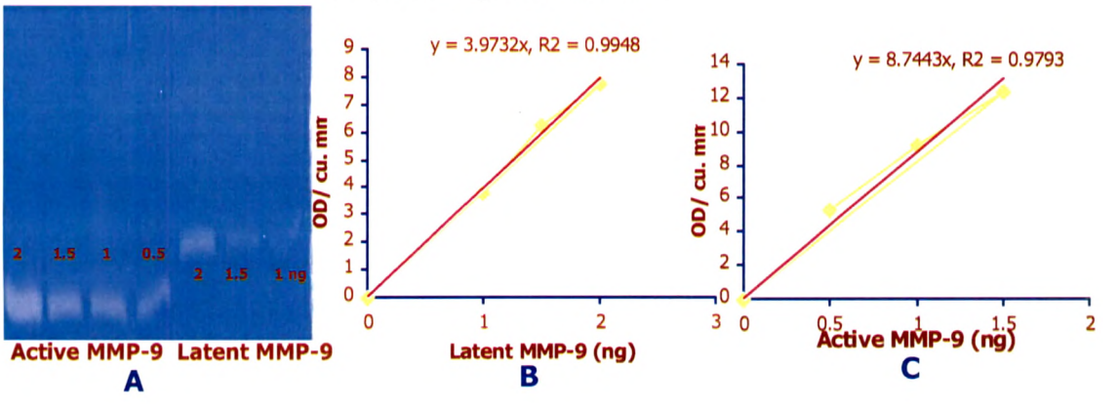
Clinico-pathological parameters	Latent MMP-2		Active MMP-2		Total MMP-2		Activation ratio MMP-2	
	F value	'p' value	F value	'p' value	F value	'p' value	F value	'p' value
Age	0.310	0.582	2.521	0.124	1.675	0.206	1.768	0.194
Sex	0.275	0.604	0.049	0.827	0.028	0.869	1.545	0.22
Habit	0.298	0.589	0.595	0.447	0.024	0.877	1.041	0.316
Tumor Differentiation	2.077	0.161	0.220	0.643	0.273	0.606	2.078	0.161
Nuclear Grade	0.362	0.700	0.146	0.865	0.331	0.721	0.068	0.935
Lymph-Node metastasis	0.009	0.926	2592	0.122	1.215	0.282	1.399	0.249
Tumor size	0.251	0.621	0.235	0.632	0.000	0.991	1.789	0.193
Stage of the disease	0.069	0.976	1.772	0.179	0.695	0.564	1.434	0.257
Early/advanced stage	0.068	0.797	0.266	0.610	0.222	0.641	0.001	0.982
Lymphatic response	3.114	0.096	0.015	0.905	4.072	0.06	1.033	0.324
Tumor infilteration	0.000	0.986	0.164	0.689	0.056	0.815	0.322	0.576

Expression of tissue gelatinase-B (MMP-9) in oral cancer

Gelatinase-B or MMP-9 is also implicated with aggressive behaviour of many solid cancers [Wood house et al., 1997]. Both the latent as well as active forms of MMP-9 were analysed by gelatin zymography. Concentration of MMP-9 was expressed as ng/50μg protein using latent and active human MMP-9 standards (Calbiochem). The representative zymogram and standard graph for latent and active MMP-9 are shown in **Figure 4.1.20.** The standards (0-2ng) of latent and active forms of MMP-9 were subjected to gelatin zymography and the positions of both forms obtained are shown in the zymogram (**Figure 4.1. 20 A**). The gels were densitometrically analysed and standard graph was plotted for concentration of standard against optical density per unit area. It yielded a linear correlation between standard concentration and activity of MMP-9 as plotted in graph. **Figure 4.1. 20 B**

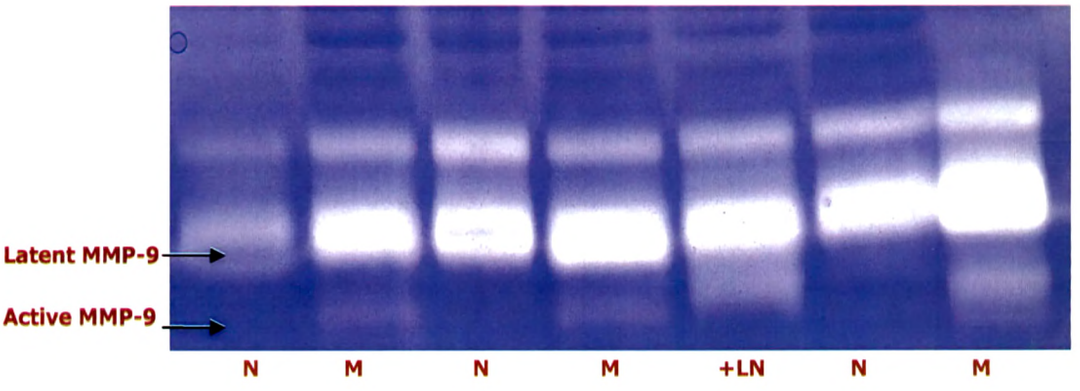
and **Figure 4.1. 20 C** show the linear standard graph for latent MMP-9 and active MMP-9 respectively which were used for analysis of unknown concentration.

Figure-4.1.20: Representative gelatin zymogram and standard curves for gelatinase-B [MMP-9]: (A; zymogram, B; latent, C; active) standards



Representative zymogram for MMP-9 expression in cytosolic preparation of oral SCC tissue samples of cancer patients is illustrated in **figure-4.1.21**. Both latent as well as active MMP-9 are expressed in adjacent normal, malignant and lymph-node positive tissues as evident from the **figure-4.1.21** showing their respective positions as clear zones exhibiting their gelatinolytic activity.

Figure-4.1.21: Representative pattern for gelatinase-B (MMP-9) in oral SCC tissues



N=Adjacent normal; **M**=malignant; +**LN**= positive lymph-node tissue

Interestingly the expression of MMP-9 is more prominent in malignant tissues compared to its normal counterpart i.e adjacent normal oral tissues. In addition to the active and latent MMP-9 forms, total and activation ratio was also calculated for MMP-9 in these tissues, which was further compared between adjacent normal and malignant tissues of the patients. Total MMP-9 was expressed as sum of latent and active forms of MMP-9 while activation ratio for MMP-9 was expressed as active MMP-9 divided by total MMP-9.

Comparison of latent, active, total and activation ratio of MMP-9 in oral cancer

Paired and unpaired student's *t*-test was performed to compare the levels of different forms of MMP-9 expressed as mean± S.E.M in adjacent normal and malignant tissues of oral cancer patients (**Table-4.1.24**).

It is clearly evident from the **Figure-4.1.22**, that the levels of all the forms were higher in malignant tissues as compared to adjacent normal tissues. The mean±S.E.M value for latent and active MMP-9 in adjacent normal tissues of oral cancer patients is 5.1575±0.3075 and 1.0403±0.1958 respectively. While the mean±S.E.M value for latent and active MMP-9 in malignant tissues of oral cancer patients is 4.9871±0.3701 and 1.8136±0.2492 respectively. Latent MMP-9 was higher in adjacent normal as compared to malignant tissues but no significant change was seen (p=0.079).

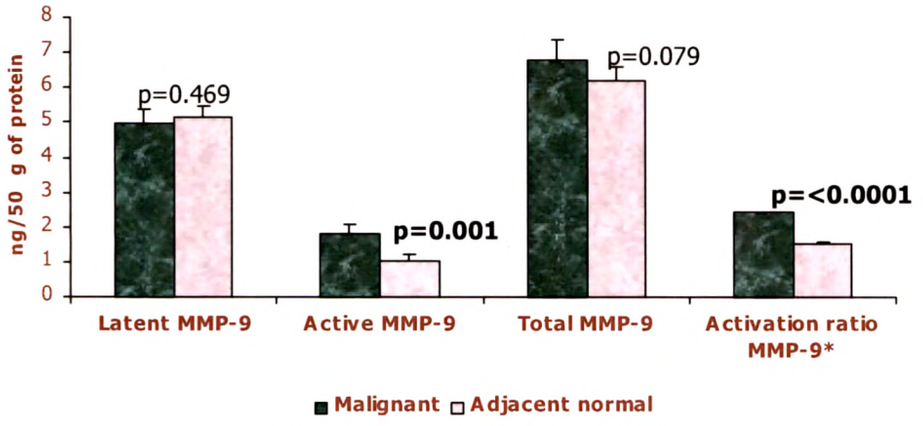
Table-4.1.24: Comparison of mean values of different forms of gelatinase-B (MMP-9) in oral SCC tissues

Biomarkers	**Oral Cancer Tissues**		**Student's *t*-test**
	Adj. normal	**Malignant**	**'p' value**
Latent MMP-9	5.1575±0.3075	4.9871±0.3701	0.725[#] 0.469[$]
Active MMP-9	1.0403±0.1958	1.8136±0.2492	**0.018**[#] **0.001**[$]
Total MMP-9	6.1978±0.3943	6.8007±0.5499	0.377[#] 0.079[$]
Activation ratioMMP-9	0.1542±0.02521	0.2433±0.0269	**0.019**[#] **<0.0001**[$]

[#]=Unpaired student's *t*-test [$]= Paired student's *t*-test

Total MMP-9 was higher in malignant tissues as compared to adjacent normal tissues. Active MMP-9 and activation ratio of MMP-9 were significantly higher in malignant tissues as compared to adjacent normal tissues (p=0.018 unpaired *t*-test, p=0.001 paired *t*-test) and (p=0.019, unpaired *t*-test; p=>0.001, paired *t*-test) respectively.

Figure-4.1.22: Comparison of latent, active, total and activation ratio of gelatinase-B (MMP-9) in oral SCC tissues



Activation ratio MMP-9* =values divided by 10

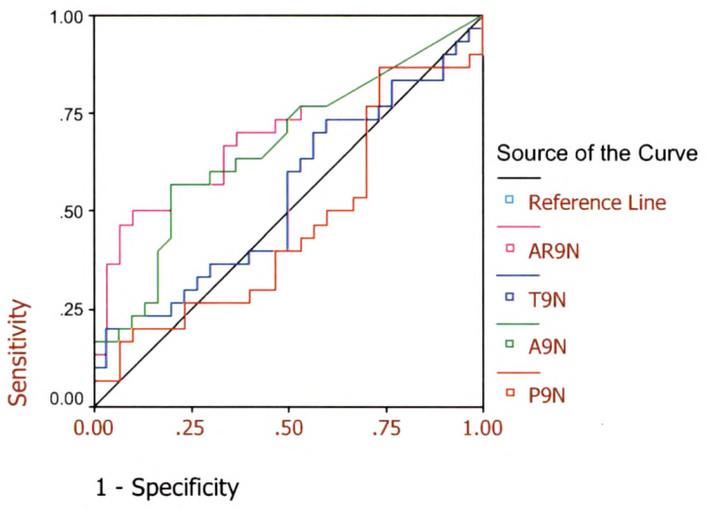
Receiver's Operating Characteristic (ROC) curve analysis

Receiver's Operating Characteristic (ROC) curve analysis was plotted to evaluate the efficiency of MMP-9 (**Figure-4.1.23**). ROC curve analysis showed that active, total MMP-9 and activation ratio of MMP-9 have good efficacy to discriminate between adjacent normal and malignant tissues using zymography technique. The area under the curve for ROC is tabulated in **Table-4.1.25** showing p value significance for all the forms of MMP-9.

Table-4.1.25: Area under ROC curve for all forms of gelatinase-B (MMP-9) in oral cancer patients

BIOMARKERS	AREA UNDER THE CURVE	'P' VALUE	*95% CI*	
			Lower bound	*Upper bound*
Latent MMP-9	0.467	0.657	0.317	*0.616*
Active MMP-9	0.661	**0.032**	0.522	*0.800*
Total MMP-9	0.539	0.605	0.391	*0.687*
Activation ratio MMP-9	*0.703*	***0.007***	*0.569*	*0.838*

Figure-4.1.23: ROC curve for gelatinase-B (MMP-9) in oral cancer patients



Comparison of gelatinase-B (MMP-9) with the clinico-pathological parameters in oral cancer patients: Clinical details like age, gender, tobacco habit, tumor differentiation, nuclear grade, lymph node status, lymphatic response, tumor infilteration, tumor size, stage of the disease were associated with the expression of MMP-9 in adjacent normal and malignant tissues of oral cancer patients. The expressions of all forms of MMP-9 in adjacent as well as malignant tissues were comparable with the clinical details. Expression of active MMP-9 in adjacent normal tissues was found to be higher in larger tumor size (T3+T4) (p=0.033) as compared to those with smaller tumor size (T1+T2) shown in Table-4.1.26. Moreover, expression of active MMP-9, total and activation ratio for MMP-9 in malignant tissues were significantly higher in oral cancer patients with larger tumor size as compared to those with smaller tumor size with p-value significance p=0.04, p=0.046 and p=0.044 respectively. Further, multivariate analysis was carried out to determine the association of gelatinase-B (MMP-9) with various clinico-pathological parameters (**Table-4.1.27**).

Table-4.1.26: Association of gelatinase-B (MMP-9) with tumor size in oral cancer patients.

Biomarkers	Tumor size Mean± S.E.M ng/50 µg		Student's *t*- test
Malignant tissues	**T1+T2**	**T3+T4**	**'p' value**
Latent MMP-9	4.597±0.35226	5.6646±0.6093	0.143
Active MMP-9	1.3210±0.4208	2.4063±0.2605	**0.04**
Total MMP-9	5.9179±0.6127	8.0709±0.8196	**0.046**
Activation ratio MMP-9	0.1858±0.0504	0.3035±0.01796	**0.044**
Adjacent normal tissues			
Active MMP-9	0.6141±0.2447	1.4723±0. 2909	**0.033**

Table-4.1.27: Association of gelatinase-B (MMP-9) in malignant tissues of oral SCC patients with clinico-pathological parameters in oral cancer patients by Multivariate analysis.

Clinico-pathological parameters	Latent MMP-9		Active MMP-9		Total MMP-9		Activation ratio MMP-9	
	F value	'p' value	F value	'p' value	F value	'p' Value	F value	'p' value
Age	0. 206	0.654	1.441	0.240	0.715	0.405	0.543	0.467
Sex	0.504	0.484	0.080	0.779	0.367	0.550	0.019	0.893
Habit	0.015	0.902	0.045	0.833	0.000	0.990	0.003	0.957
Tumor Differentiation	2.653	0.115	0.032	0.858	1.363	0.253	0.328	0.572
Nuclear Grade	0.431	0.655	2.316	0.119	1.099	0.349	1.409	0.111
Lymph-Node metastasis	1.469	0.238	0.988	0.331	1.805	0.193	0.001	0.971
Tumor size	2.121	0.157	5.094	**0.033**	4.205	**0.051**	5.384	**0.028**
Stage of the disease	1.271	0.307	5.390	**0.006**	3.090	**0.046**	5.989	**0.003**
Early/advanced stage	2.883	0.101	8.267	**0.008**	6.280	**0.019**	7.106	**0.013**
Lymphatic response	0.499	0.489	2.647	0.122	1.394	0.254	2.151	0.161
Tumor infilteration	0.532	0.473	2.155	0.155	1.316	0.263	3.297	**0.082**

Expression of active MMP-9, total MMP-9 and activation ratio of MMP-9 in malignant tissues correlated significantly with tumor size, different stages of the disease and early and advanced stage of the disease in oral cancer patients. However expression of latent, active, total and activation ratio of MMP-9 in adjacent normal as well as malignant tissues did not significantly associate with other clinicopathological parameters.

Expression of gelatinases in lymph node tissues:

Expression of gelatinases was also analysed from lymph node positive and lymph node negative tissues. **Table-4.1.28** shows the comparison of latent, active, total and activation ratio for MMP-2 and MMP-9 in lymph node positive and negative tissues. Latent MMP-2 (p=0.099), active MMP-2 (p=0.01), and active MMP-9 (p=0.804) was higher in lymph-node positive tissues as compared to lymph node negative tissues.

Table-4.1.28: Comparison of latent and active forms of gelatinase-A (MMP-2) and -B (MMP-9) in oral lymph node tissues

Biomarkers	Oral Cancer Tissues		Paired student's *t*-test
	Positive Lymph-node	Negative Lymph-node	'p' value
Latent MMP-2	14.3929±1.9464	10.8693±1.3835	0.099
Active MMP-2	9.4294±1.7437	3.5957±1.425	**0.01**
Latent MMP-9	5.6531±0.9599	5.6806±0.488	0.976
Active MMP-9	1.7064±0.4469	1.5922±0.3116	0.804

Comparison of gelatinase-A and gelatinase-B in oral SCC patients

All the adjacent as well as malignant tissues of oral SCC patients showed expression of gelatinases and its tissue inhibitors. MMPs are modulated and their expression is controlled by other MMPs and TIMPs either in process of activation or inhibition. Literature reviews expression of both gelatinases modulating the action of each other, hence Bivariate correlations like Pearsons and Spearmans rho correlation were calculated for comparing latent and active forms of both MMP-2 and MMP-9 in malignant tissues of oral SCC patients (**Table-4.1.29**).

A positive correlation was observed between latent and active forms of both MMP-2 and MMP-9 in tissues. Latent MMP-9 correlated significantly with active MMP-9, latent MMP-2 and active MMP-2. Active MMP-9 correlated positively with latent and active MMP-2, but significantly with latent MMP-9. Latent MMP-2 correlated positively with active MMP-9, and significantly with latent MMP-9 and active MMP-2. While active MMP-2 correlated positively with Latent MMP-9, and significantly with latent MMP-9 and active MMP-9.

Table-4.2.29 Correlation of tissue levels of latent and active forms of gelatinase-A (MMP-2), gelatinase-B (MMP-9) in malignant tissues of oral cancer patients

Factors	Pearsons correlation	Significance Two-tailed	Spearmans Rho correlation	Significance Two-tailed
Latent MMP-9				
Active MMP-9	0.560	**0.001**	0.508	**0.004**
Latent MMP-2	0.719	**>0.0001**	0.676	**<0.0001**
Active MMP-9	0.491	**0.006**	0.405	**0.027**
Active MMP-9				
Latent MMP-9	0.560	**0.001**	0.508	**0.004**
Latent MMP-2	0.202	0.285	0.158	0.404
Active MMP-2	0.325	0.080	0.287	0.124
Latent MMP-2				
Latent MMP-9	0.719	**>0.0001**	0.676	**<0.0001**
Active MMP-9	0.202	0.285	0.158	0.404
Active MMP-2	0.400	**0.028**	0.448	**0.013**
Active MMP-2				
Latent MMP-2	0.491	**0.006**	0.405	**0.027**
Latent MMP-9	0.325	0.080	0.287	0.124
Active MMP-9	0.400	**0.028**	0.448	**0.013**

Moreover, since, both gelatinases utilize similar substrate-gelatin and cleave the type-IV collagen in tissues, it will be interesting to assess which prototype of gelatinase predominates in oral tissues of these cancer patients. Both forms of gelatinases, MMP-2 and MMP-9 were compared in adjacent normal and malignant oral tissues. **Table-4.1.30** shows the comparison of latent, active, total and activation ratio of MMP-2 and MMP-9 in adjacent normal and malignant tissues. In adjacent normal tissues, latent MMP-2 (p=<0.0001), active MMP-2 (p=0.024) and total MMP-2 (p=<0.0001) were significantly elevated as compared to latent, active and total MMP-9. While in malignant oral tissues, the expression of all forms latent, active, total and activation ratio of MMP-2 were significantly higher as compared to all forms of MMP-9 expression (p=<0.0001).

Table-4.1.30 Comparison of mean values of gelatinase-A (MMP-2) and gelatinase-B (MMP-9) in oral SCC tissues

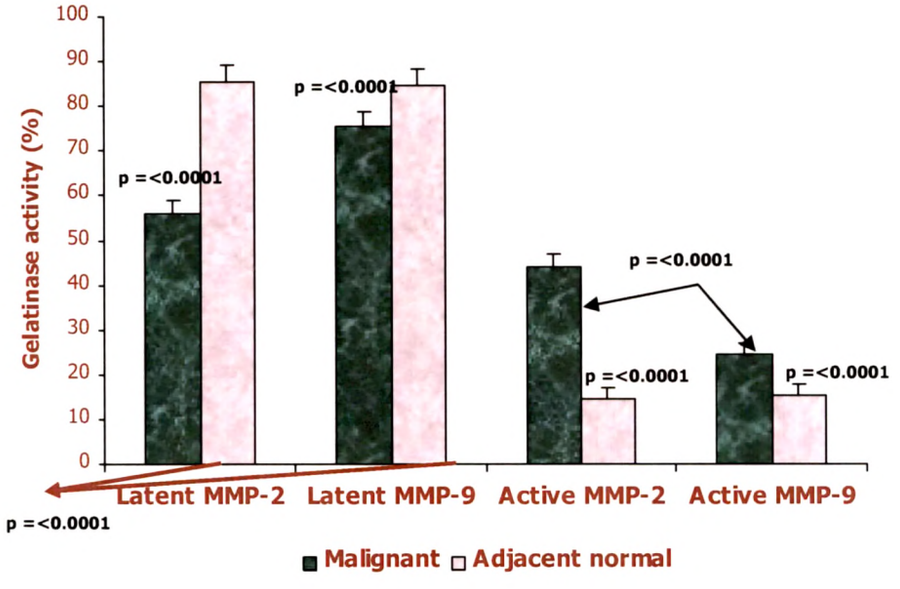
Biomarkers	Adjacent normal tissues Mean± S.E.M ng/50 μg		Paired student t- test 'p' value
	MMP-2	MMP-9	
Latent form	13.4472±0.9314	5.1575±0.3075	**<0.0001**
Active form	2.9983±0.8527	1.0403±0.1958	**0.024**
Total	16.4455±1.3080	6.1978±0.3943	**<0.0001**
Activation ratio	0.1446±0.03640	0.1542±0.02521	0.797
	Malignant tissues		
	MMP-2	MMP-9	
Latent form	13.6590±0.9677	4.987088±0.3701	**<0.0001**
Active form	11.3710±1.0304	1.8136±0.2492	**<0.0001**
Total	25.0300±1.6722	6.8007±0.5499	**<0.0001**
Activation ratio	0.4406±0.03057	0.2433±0.02690	**<0.0001**

Further percentage gelatinase activity of latent and active forms of both MMP-2 and MMP-9 was calculated considering total activity of the respective MMPs as 100%. **Table-4.1.31** shows the comparison of percentage activity of both latent and active MMP-2 and MMP-9 between adjacent normal and malignant tissues and also between the two active gelatinases in both adjacent normal and malignant tissues. The percentage activity of both latent MMP-2 and latent MMP-9 was higher (p=<0.0001) in adjacent normal tissues as compared to malignant tissues, while percentage activity of both active MMP-2 and active MMP-9 was significantly higher (p=<0.0001) in malignant tissues as compared to adjacent normal tissues. Interestingly, when the percentage activity in respective tissues was compared, it was observed that in adjacent normal tissues percentage activity of latent MMP-2 was higher than latent MMP-9, while active MMP-9 was higher than active MMP-2, but with no significant change was seen (p=0.797 each) (**Table-4.1.31**). However, in malignant tissues the percentage activity of latent MMP-2 was significantly lower (p=<0.0001) than latent MMP-9 while the percentage activity of active MMP-2 was significantly higher (p=<0.0001) than active MMP-9 as depicted in **Figure-4.1.24**.

Table-4.1.31 Comparison of percentage activity of gelatinase-A (MMP-2) and gelatinase-B (MMP-9) in oral SCC tissues

Biomarkers	Percentage activity Mean± S.E.M (%)		Paired Student's *t*- test
	Adj. normal	**Malignant**	**'p' value**
Latent MMP-2	85.5441±3.6404	55.9445±3.0567	**<0.0001**
Active MMP-2	14.4559±3.6404	44.0555±3.0567	**<0.0001**
Latent MMP-9	84.5835±2.5211	75.6715±2.6901	**<0.0001**
Active MMP-9	15.4165±2.5211	24.3285±2.6901	**<0.0001**
Oral Tissues	**Latent MMP-2**	**Latent MMP-9**	
Adj. normal	85.5441±3.6404	84.5835±2.5211	0.797
Malignant	55.9445±3.0567	75.6715±2.6901	**<0.0001**
Oral Tissues	**Active MMP-2**	**Active MMP-9**	
Adj. normal	14.4559±3.6404	15.4165±2.5211	0.797
Malignant	44.0555±3.0567	24.3285±2.6901	**<0.0001**

Figure-4.1.24 Comparison of percentage activity of gelatinase-A (MMP-2) and gelatinase-B (MMP-9) between malignant and adjacent normal oral SCC tissues



SECTION 4.2: EXPRESSION OF CIRCULATORY BIOMARKERS IN ORAL CANCER PATIENTS

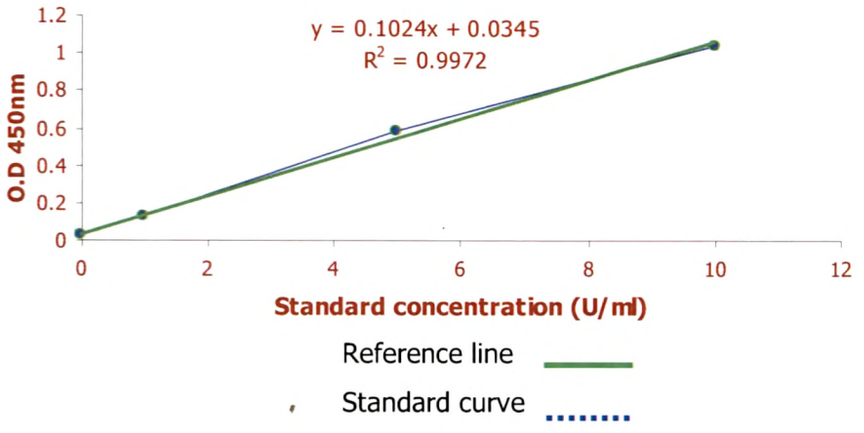
Biomarkers help in finding out the stage of the disease during prognostication and also assist in treatment monitoring. The present study is therefore aimed to monitor blood samples by non-invasive method to determine parameters of oral cancer and to evaluate their association with tumor progression. This would enable us to assess their usefulness as follow-up parameters in these patients. Twenty nine oral cancer patients were followed up during their course of treatment and their blood samples were classified into complete responders (n=44) and non-responders (n=9) according to the clinical details obtained for the patients during their follow-up. These untreated patients (samples collected at time of diagnosis with history of no previous treatment taken) were monitored for circulatory biomarkers throughout their follow-up. Serum and plasma were separated for assessing and evaluating biomarkers in these oral cancer patients.

4.2.1. Serum p53 autoantibody analysis

Tumor suppressor gene p53 is the most commonly altered specific genetic target involved in human malignant transformation. Loss of Its function has been associated with varied events like defective DNA repair, increased genetic instability, survival of cells with increased mutational load involved in progression of cancer [Kastan et al., 1991; Levin, 1997]. p53 is a tumor suppressor protein which, when active, regulates genes related to cell cycle regulation, DNA repair and apoptosis. Although wild type p53 is present in normal tissues and cells, its short half-life (5-20min) makes its expression almost undetectable in healthy normal tissues [Giaccia and Kastan 1998]. Mutated p53 gene leads to production of autoantibodies against accumulated mutant p53 protein in circulation. Upon stimulation, p53 gets stabilized so that its expression can be detected with anti-p53 antibodies using standard ELISA techniques. Using ELISA technique, circulating levels of p53 autoantibodies were measured in serum samples of controls and oral cancer patients. . With each batch of the assay, anti p53 anti-antibody standards

were run. **Figure-4.2.24** shows the standard curve plotted for p53 autoantibodies to calculate unknown levels in serum samples of the subjects.

Figure-4.2.24: Standard curve for anti serum p53 antibodies



All the subjects were classified into two groups as sero-positive and sero-negative depending upon the presence or absence of serum p53 autoantibodies respectively. **Table-4.2.32** depicts frequency of positivity of serum p53 autoantibodies in the subject groups. Presence of serum p53 autoantibodies was seen in about 19.23% oral cancer patients, while controls showed complete absence of p53 autoantibodies in the serum.

Table-4.2.32: Frequency of serum p53 autoantibodies in subjects

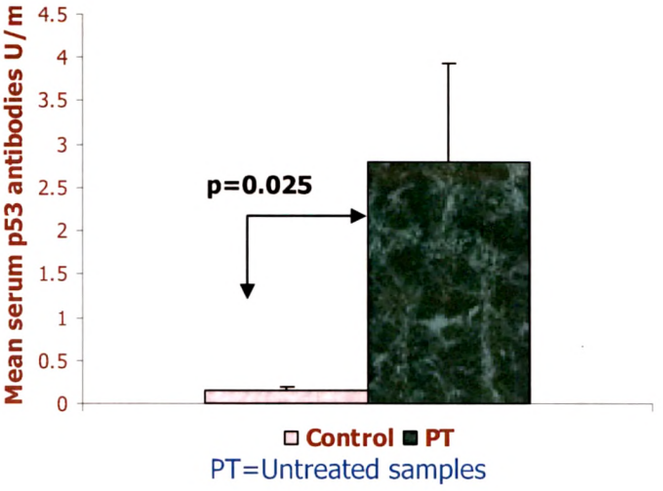
Subjects	Sero-positive	Sero-negative
Controls (N=100)	0%	100%
Oral cancer patients (N=120)	19.23%	80.77%
Follow-up (N=53)	23.7%	76.3%

Oral cancer patients showed significantly higher mean values than the controls for p53 autoantibodies with unpaired student's *t*-test with significance of p=0.025 (**Table-4.2.33**). **Figure-4.2.25** shows the comparison of mean values of serum p53 autoantibodies among the subjects.

Table-4.2.33: Mean values of serum p53 autoantibodies in subjects

Subjects	Serum p53 autoantibodies (Mean± S.E.M)	'p' value
Control	0.158±0.0363	**0.025**
Oral cancer patients	2.794±1.136	

Figure-4.2.25: Comparison of mean values of serum p53 auto antibodies in controls and oral cancer patients



Receiver's Operating Characteristic Curve analysis

Receiver's Operating Curve (ROC) was plotted to determine the discriminatory efficacy of serum p53 autoantibodies between controls and cancer patients. The area under the curve for the ROC analysis showing 95% CI and statistical significance of the test for serum p53 autoantibodies among subjects is tabulated in **Table-4.2.34**. The ROC analysis revealed that the serum p53 autoantibodies had a good efficacy to discriminate between controls and oral cancer patients as documented by area under the curve (**Figure-4.2.26**).

Figure-4.2.26: ROC curve for serum p53 autoantibodies in controls vs oral cancer patients

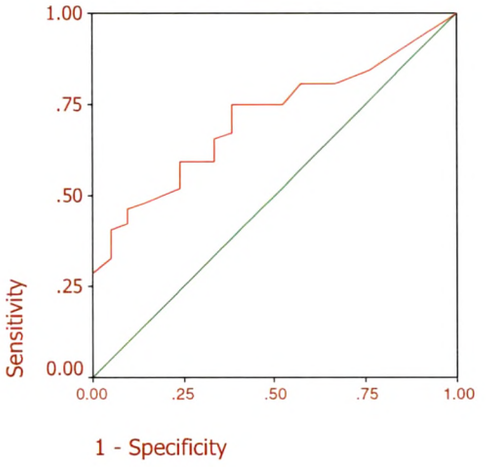


Table-4.2.34: Area under ROC curve for serum p53 autoantibodies in subjects (controls vs oral cancer patients)

Biomarker	Area under the curve	'p' value	95% CI	
			Lower bound	Upper bound
Serum p53 autoantibodies	0.712	**0.005**	0.594	0.831

Comparison of serum p53 autoantibodies in the subjects associated with tobacco habit:

Association of tobacco habits and p53 antibodies was also evaluated. The frequency as well as mean values of serum p53 autoantibodies were compared between subjects having habit of tobacco (WHT), those with no habit of tobacco (NHT) and also among the oral cancer patients. Subjects with NHT were 100% sero-negative for serum p53 autoantibodies in both healthy controls and oral cancer patients. Whilst subjects with WHT only 21.74% of oral cancer patients were sero-positive for serum p53 autoantibodies as illustrated in table-4.2.35. It was observed that oral cancer patients with tobacco habits showed presence, while those with no habit of tobacco showed complete absence of serum p53 autoantibodies.

Table-4.2.35: Frequency and association of serum p53 autoantibodies with tobacco habits in subjects

Subjects	Tobacco habit		No habit	
Serum p53 autoantibodies	Sero-positive	Sero-negative	Sero-positive	Sero-negative
Controls	Nil	100%	Nil	100%
Oral cancer patients	21.74%	78.26%	Nil	100%

As shown in the **Table-4.2.36**, it was observed that the mean levels of serum p53 autoantibodies were significantly higher in oral cancer patients (p=0.022) having habit of tobacco (WHT) as compared to healthy controls. In non-tobacco users (NHT), no significant change was observed in the mean levels of p53 autoantibodies. Moreover the mean levels of serum p53 autoantibodies were also significantly higher in oral cancer patients (p=0.036) with WHT as compared to NHT (**Table-4.2.36**).

Table-4.2.36: Mean values of serum p53 autoantibodies among tobacco users and nonusers of the subjects

Subjects	Serum p53 autoantibodies Mean± S.E.M	'p' value
Tobacco habit (WHT)		
Controls	0.08857±0.0478	**0.022**
Oral cancer patients	3.1166±1.278	
No habit of tobacco (NHT)		
Controls	0.1555±0.042	0.188
Oral cancer patients	0.3217±0.1057	
Oral cancer patients		
Tobacco habit (WHT)	3.1166±1.278	**0.035**
No habit of tobacco (NHT)	0.32174±0.105	

Comparison of serum p53 autoantibodies in the subjects associated with clinico-pathological parameters.

The clinical details of the oral cancer patients were recorded which help to assess and determine the status of disease like stage, tumor bulk and eventually its severity. Serum p53 autoantibodies was correlated with the clinico-pathological parameters like age, sex, habit of tobacco, tumor size and differentiation, nuclear grade, lymph node involvement, lymphatic response, tumor infilteration and stage of the disease of the oral cancer patients. Frequency of sero-positivity for p53 autoantibodies was associated with the clinical details as summarized in **Table-4.2.37.** Serum p53 autoantibodies were present in 26.66% of patients with (T1+T2) tumor size and 47.61% of patients with larger tumor size (T3+T4). The presence of serum p53 autoantibodies with higher levels was found to be more prominent in advanced stage of patients showing its importance in disease progression (**Table-4.2.38**). It was present in 22.73% of patients with lymph-node metastasis. 27.5% and 19.44% of oral cancer patients with well differentiated and moderately differentiated tumor were observed respectively. 28.57% and 18.52% of tumors of nuclear grade I and II showed the presence of p53 autoantibodies respectively.

Table-4.2.37: Frequency of serum p53 autoantibodies in the oral cancer patients

Clinical details	Serum p53 antibodies		Seropositivity
	Positive	Negative	(%)
Tumor size			
T1	2/22	20/22	9.1%
T2	3/19	16/19	15.79%
T3	1/5	4/5	20%
T4	9/22	13/22	40.9%
Tumor differentiation			
Well	11/40	29/40	27.5%
Moderate	14/72	58/72	19.44%
Poor	0/8	0/8	0%
Nuclear grade			
I	6/21	15/21	28.57%
II	15/81	66/81	18.52%
Lymph-node metastasis			
Yes	10/44	34/44	22.73%
No	9/39	30/39	23.08%
Tumor infilteration			
Yes	6/56	26/56	18.75%
No	3/14	4/14	42.86%
Stage of the disease			
Early (I+II)	0/28	7/28	0%
Advanced (III+IV)	23/92	27/92	25%

Table-4.2.38: Comparison of mean values of serum p53 autoantibodies between tumor size and stage of the disease

Oral cancer patients	Serum p53 autoantibodies Mean± S.E.M	'p' Value
Size of the tumor		0.063
T1+T2	0.3688±.1261	
T3+T4	4.5280± 2.1287	
Stage of the disease		
Early	0.2686±0.09972	0.054
Advanced stage	3.2681± 1.5045	

Further the association of these clinico-pathological parameters was determined using univariate analysis (**Table-4.2.39**). Presence of serum p53 autoantibodies was significantly associated with only lymph node metastasis but not with other clinico-pathological factors.

Table-4.2.39: Univariate analyses for presence of serum p53 autoantibodies

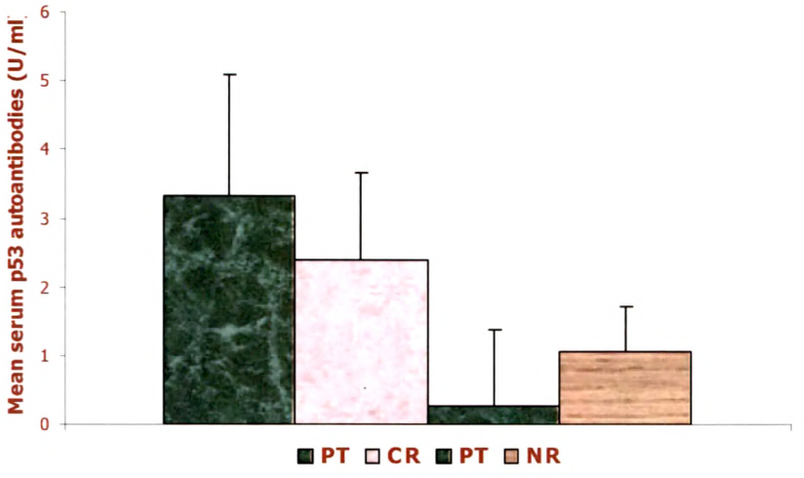
Clinico-pathological parameters	Serum p53 autoantibodies	
	F value	'p'value
Age	1.145	0.290
Sex	0.024	0.878
Habit	0.612	0.438
Tumor Differentiation	0.074	0.781
Nuclear Grade	0.363	0.551
Tumor size	0.777	0.516
Lymphatic response	0.031	0.864
Tumor infilteration	0.016	0.900
Lymph Node metastasis	1.786	**0.004**
Stage of the disease	0.276	0.842
Early/ advanced stage	0.758	0.389

Association of serum p53 autoantibodies with the course of disease (Untreated levels and follow-up levels)

Serum p53 antibodies association with the disease course was studied by comparing the untreated levels with the levels in follow-up serum samples collected from the oral cancer patients. These patients were monitored for p53 autoantibodies status throughout their follow-up. Seropositivity of p53 autoantibodies was observed in 23.7% of follow-up patients.

Table-4.2.40 shows the comparison of mean values of serum p53 autoantibodies between untreated oral cancer patients (PT) and their complete responders (CR). The mean values were higher in untreated oral cancer patients (p=0.297) as compared to their complete responders. While the mean values of serum p53 autoantibodies were lower in untreated oral cancer patients (p=0.297) as compared to the serum levels of the non-responder (NR) patients (**Figure-4.2.27**).

Figure-4.2.27: Comparison of serum p53 autoantibodies levels between untreated oral cancer patients and their follow-up



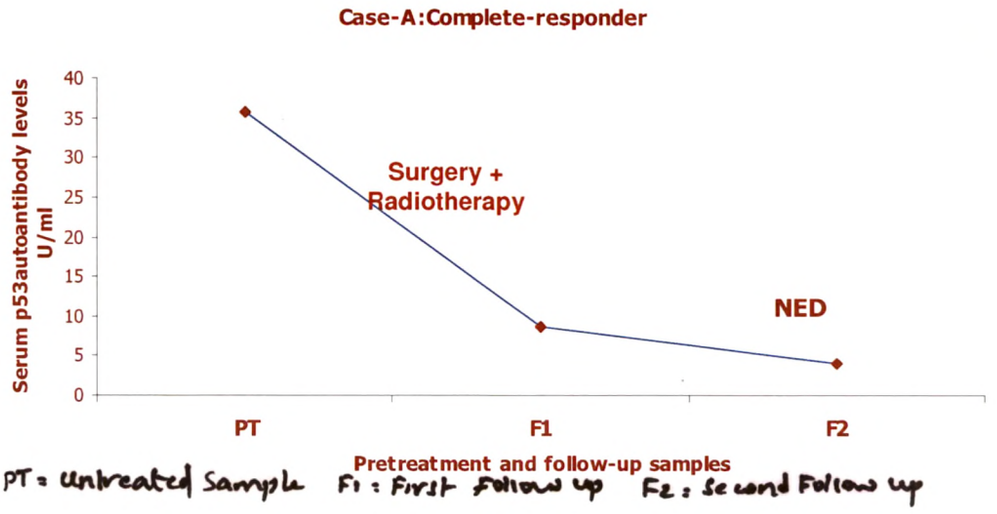
PT=untreated samples **CR**=Complete-Responders **NR**=Non-responders

Table-4.2.40: Comparison of Mean values of serum p53 autoantibodies between untreated and follow-up of the oral cancer patients

Oral cancer patients	Serum p53 autoantibodies Mean± S.E.M	Trend	'p' value
Untreated oral cancer patients (PT)	3.317±1.769	Higher	0.360
Complete responders (CR)	2.401±1.269		
Untreated oral cancer patients (PT)	0.272±1.111	Lower	0.297
Non-responders (NR)	1.056±0.659		

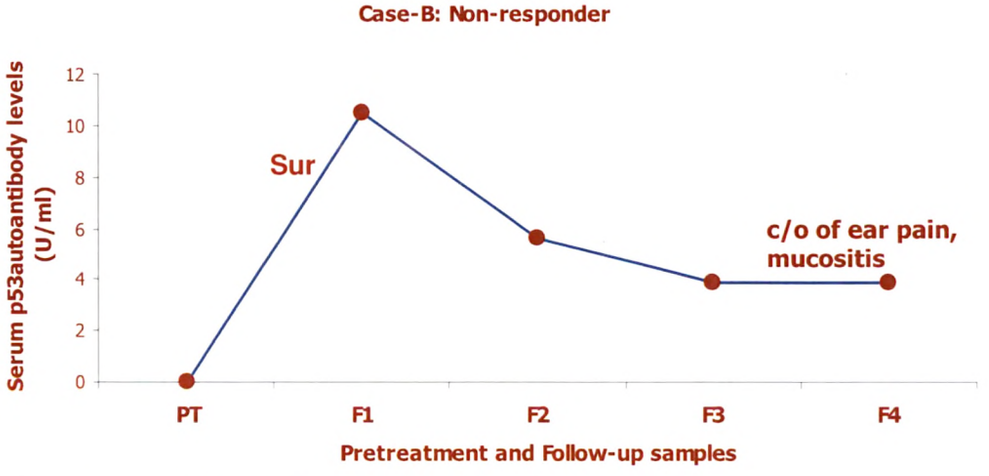
The oral cancer patients were followed up and monitored for p53 status and their representative cases of complete-responders and non-responders and the results are shown in **Figures-4.2.28** and **Fig-4.2.29.** In case-A, the oral cancer patient is a 25 yr old chronic alcoholic and tobacco user (chewing and snuffing) male suffering from squamous cell carcinoma of right buccal mucosa diagnosed at stage IV. Serum p53 autoantibodies were present before any anticancer treatment as well as during follow-up. The mean p53 levels before treatment was 35.76 U/ml. The patient received surgery and radiotherapy treatment and during subsequent follow-up, levels of serum p53 autoantibodies declined to 8.733U/ml and 3.94U/ml reported with clinical report indicating locally no evidence of disease (NED) showing response to treatment.

Figure-4.2.28 Representative case for Complete-responders (CR)



Case-B is a representative of non-responder of 34 yrs old male patient chronic alcohol and tobacco user, diagnosed with cT3N1Mx squamous cell carcinoma of left buccal mucosa showing comparable levels of p53 autoantibodies. The patient was seronegative with mean 0 values before treatment but during subsequent follow-up, the p53 autoantibody levels were detected with presence of antibody in the serum at the levels 10.526 and later 5.618 U/ml, which remained persistent during subsequent follow-up. The patient had complaint of pain at local site and ear with mucositis and showed no response to therapy.

Figure-4.2.29 Representative case for Non-responders (NR)



PT=Untreated sample; F1= first follow-up sample; F2=second follow-up sample

4.2.2 Serum Interleukin-8 (IL-8)

IL-8 is an important angiogenic chemokine having autocrine role in modulating survival and proliferation of tumor cells in a wide variety of human solid tumors comprising head and neck squamous cell carcinoma [Ina et al., 2004; Chen Z et al., 1999]. Circulating levels of IL-8 were measured in serum samples of healthy controls and oral cancer patients. These oral cancer patients were also followed up and monitored for serum IL-8 by highly sensitive ELISA technique. With each batch of the assay, IL-8 standards were run and **Figure-4.2.30** shows the standard curve plotted for serum IL-8 to calculate unknown levels in serum samples of the subjects.

Figure-4.2.31 shows the comparison of mean values of serum IL-8 and its significance measured among the subjects by unpaired student t-test analysis. Oral cancer patients showed significantly higher mean values of serum IL-8 (p= <0.001) than the healthy controls **(Table-4.2.41)**.

Figure-4.2.30: Standard curve for serum IL-8

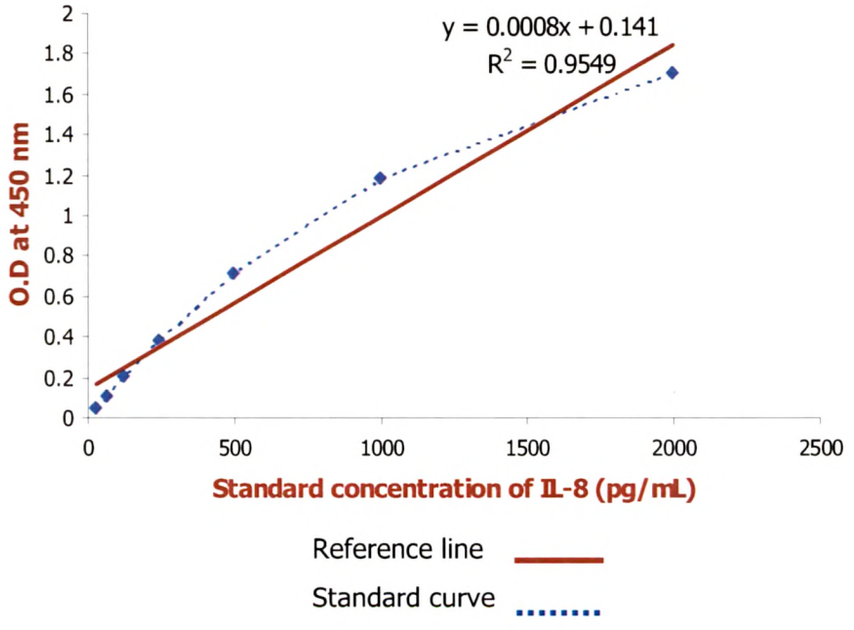
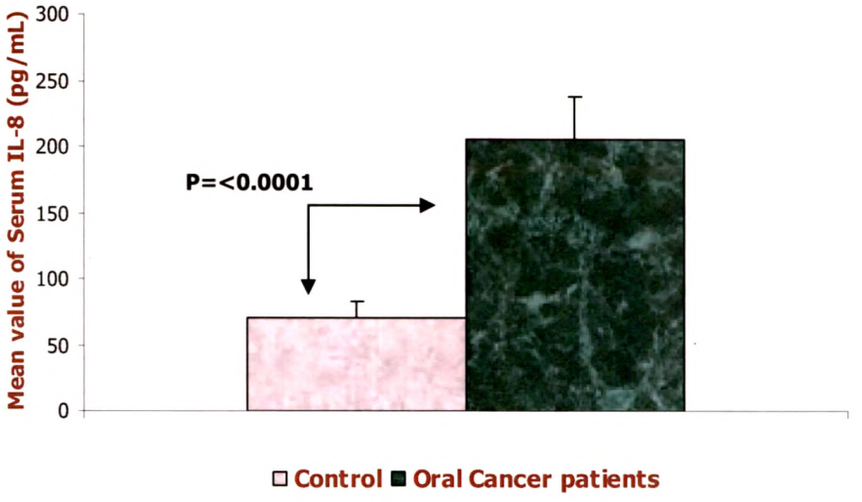


Table-4.2.41: Comparison of Mean Values of serum IL-8 in subjects

Subjects	Serum IL-8 (Mean± S.E.M)	'p' value
Control	69.79251±13.1593	<0.001
Oral cancer patients	205.5033±32.8974	

Figure-4.2.31: Comparison of mean values of serum IL-8 in controls and oral cancer patients



Receiver's Operating Characteristic Curve analysis

Receiver's Operating Curve (ROC) was also plotted to determine the discriminatory efficacy of serum IL-8 in the controls and oral cancer patients. ROC curve plot analysis and its statistical significance revealed that the serum IL-8 had good efficacy to significantly discriminate between controls and oral cancer patients as illustrated in F**igure-4.2.32** and **Table-4.2.42**.

Figure-4.2.32: ROC curve for serum IL-8 in controls vs oral cancer patients

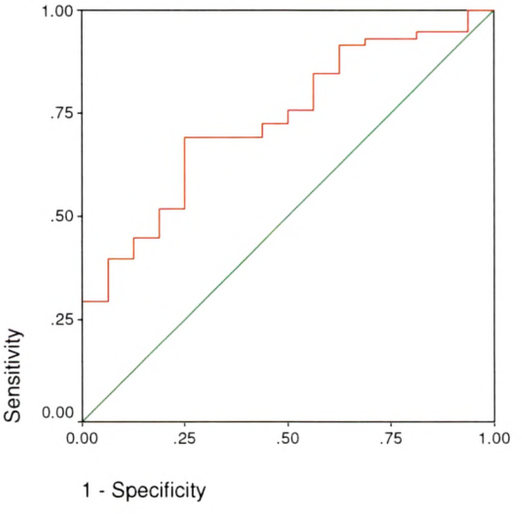


Table-4.2.42: Area under ROC curve for serum IL-8 in subjects (controls vs oral cancer patients)

Biomarker	Area under the curve	'p' value	95% CI	
			Lower bound	Upper bound
Serum IL-8	0.733	**0.005**	0.605	0.861

Comparison of serum IL-8 in the subjects associated with tobacco habit

Association of tobacco habits and serum IL-8 was also evaluated. The mean values of serum IL-8 were compared between subjects having habit of tobacco (WHT), those with no habit of tobacco (NHT) and also among the oral cancer patients. As shown in the **Table-4.2.43**, it was observed that the

Table-4.2.43: Mean values of serum IL-8 among tobacco users and nonusers of the subjects

Subjects	Serum IL-8 Mean± S.E.M	'p' value
Tobacco habit (WHT)		
Controls	54.1580±9.2099	**<0.001**
Oral cancer patients	192.6616±31.4620	
No habit of tobacco (NHT)		
Controls	81.0180±20.0610	0.177
Oral cancer patients	329.4157±161.640	
Oral cancer patients		
Tobacco habit (WHT)	192.6616±34.4620	0.436
No habit of tobacco (NHT)	329.4157±161.640	

mean levels were significantly higher in oral cancer patients with WHT as compared to healthy controls. In non-tobacco users (NHT), the mean values for serum IL-8 was higher in oral cancer patients as compared to healthy controls (p=0.177). The mean values for serum IL-8 levels were also higher in cancer patients with WHT (p=<0.0001) as compared to NHT.

Association of serum IL-8 with clinico-pathological parameters in oral cancer patients by Univariate analysis

Student's *t*-test analysis revealed that the mean values for serum IL-8 were significantly higher in cancer patients with larger tumor size, lymphatic

response, tumor infilteration and those being in stage III compared to stage II patients as illustrated from **Table-4.2.44.**

Table-4.2.44: Comparison of mean values of serum IL-8 in oral cancer patients

Oral cancer patients	Serum IL-8 Mean± S.E.M	'p' value
Size of the tumor		
T1+T2	110.048±16.824	**0.037**
T3+T4	253.620± 62.926	
Tumor infilteration		
Yes	214.649±44.962	**0.028**
No	95.420± 25.765	
Lymphatic response		
Yes	250.551±63.290	**0.019**
No	83.888± 19.881	
Stage of the disease		
Stage I	136.190±45.184	Ns
Stage II	80.795±31.464	Ns
Stage III	212.094±51.680	**0.047**
Stage IV	193.625±57.298	Ns
Stage of the disease		
Early	104.535±26.453	**0.056**
Advanced	200.294± 40.637	

Ns=non-significant

Moreover, the mean values were also higher in advanced stage (p=0.056) of patients as compared to those in early stage of the disease suggesting its role in disease progression. Further the association of these clinico-pathological parameters was also determined using Univariate analysis (**Table-4.2.45**). Serum IL-8 was significantly associated in patients with larger tumor size (p=0.05) but not with other clinico-pathological parameters.

Table-4.2.45: Univariate analyses for association of clinico-pathological parameters with the presence of serum IL-8

Clinico-pathological parameters	Serum IL-8	
	F value	'p' value
Age	0.708	0.404
Sex	2.341	0.131
Habit	1.806	0.185
Tumor differentiation	0.000	0.984
Nuclear grade	0.006	0.937
Tumor size	4.047	**0.05**
Lymphatic response	1.828	0.188
Tumor infilteration	1.277	0.266
Lymph node metastasis	2.508	0.121
Stage of the disease	0.382	0.766
Early/ advanced stage	1.044	0.313

Association of serum IL-8 with the course of disease (Untreated levels and follow-up levels)

The association of serum IL-8 with the disease course was also studied by comparing the untreated levels with the follow-ups. **Table-4.2.46** shows the comparison of mean values of serum IL-8 levels between untreated oral cancer patients (PT) and their complete responders (CR). The mean values of serum IL-8 was lower in untreated oral cancer patients as compared to their complete responders (p=0.497). While the mean values of serum IL-8 levels were higher in untreated oral cancer patients as compared to the serum levels of the non-responder (NR) patients (p=0.668).

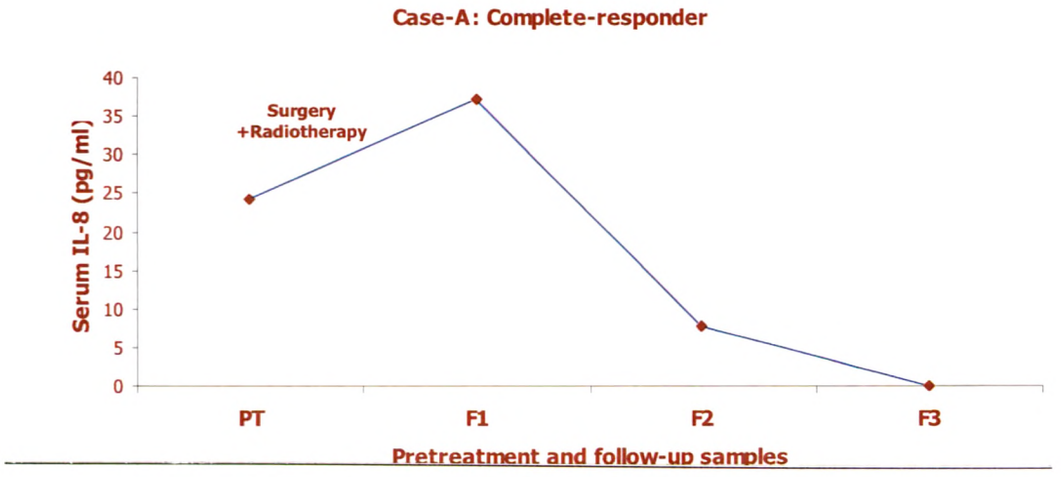
Table-4.2.46: Comparison of mean values of serum IL-8 between untreated and follow-up of the oral cancer patients

Oral cancer patients	Serum IL-8 Mean± S.E.M	Trend	'p' value
Untreated oral cancer patients (PT)	186.500±28.306		
Complete responders (CR)	239.351±87.535	Lower	0.497
Untreated oral cancer patients (PT)	131.246±22.989		
Non-responders (NR)	102.500±44.006	Higher	0.668

The oral cancer patients were followed up and monitored for serum IL-8 levels and their representative cases of complete-responder and non-responder are shown in **Figure-4.2.33** and **Figure-4.2.34.** In case-A, the

oral cancer patient is a 44 yrs old male suffering from carcinoma of left tongue diagnosed with pT1N1M0. The patient had no habit of tobacco and received Surgery and radiotherapy as anticancer therapy. The untreated serum level of IL-8 was 24.29 pg/ml. The serum samples for the first follow up were comparable with PT levels of ~37.14 pg/ml with complaint of pain. But in later two follow-ups, the patient was asymptomatic with no complaints and serum IL-8 levels declined gradually to 7.88 pg/ml and 0 pg/ml as depicted in **Figure-4.2.33**. The serum IL-8 levels were higher at the time of treatment, which declined eventually during subsequent follow-ups correlating with the good response to therapy.

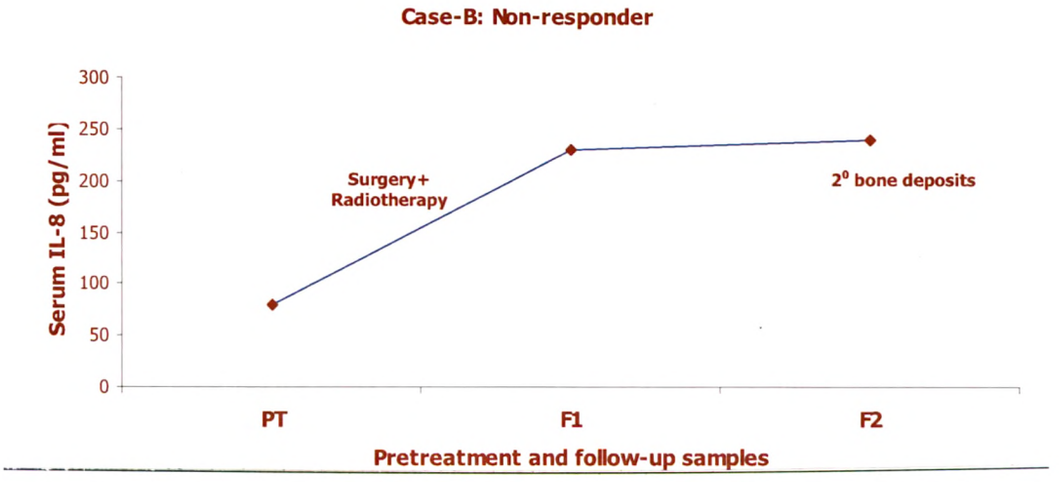
Figure-4.2.33: Representative case for complete responder (CR)



PT=Untreated sample; F1= first follow-up sample; F2=second follow-up sample

Figure-4.2.34 represents a case of a non-responder who is a 42yrs female old patient suffering from squamous cell carcinoma of right tongue. The clinical stage of the patient could not be assessed as anticancer therapy patient underwent surgery followed by radiotherapy and chemotherapy. The untreated serum level was 17.1.7 pg/ml, but during follow-up, the levels rose to 230.0 and remain persistent in later follow up as 239.0 pg/ml. The patient showed complaint of pain with nodal status positive showing no response to therapy. The increased levels of IL-8 correlated well with the disease status.

Figure 4.2.34: Representative case for non-responder (NR)



PT=Untreated sample; F1= first follow-up sample; F2=second follow-up sample

4.2.3 Serum glycoprotein constituents

Malignant transformation of cancerous cells involves molecular changes associated with altered cell surface oligosaccharide component of glycoconjugates including glycoproteins and glycolipids as the hallmark of cancer progression. Circulating markers associated with protein glycosylation were studied in oral cancer patients. Serum levels of total sialic acid (TSA), lipid bound sialic acid (LSA), mucoid protein (MP), and hexoses were determined in all the subjects including healthy individuals (controls) and oral cancer patients and their follow-up to explain their clinical significance.

Comparison of mean values of serum glycoprotein constituents in subjects: Figure-4.2.35 shows the comparison of mean values of serum glycoprotein constituents measured among the subjects by unpaired student's *t*-test analysis. Oral cancer patients showed significantly higher mean values of serum glycoprotein constituents than the healthy controls (**Table-4.2.47**).

Figure-4.2.35: Comparison of mean values of serum glycoprotein constituents in controls and oral cancer patients

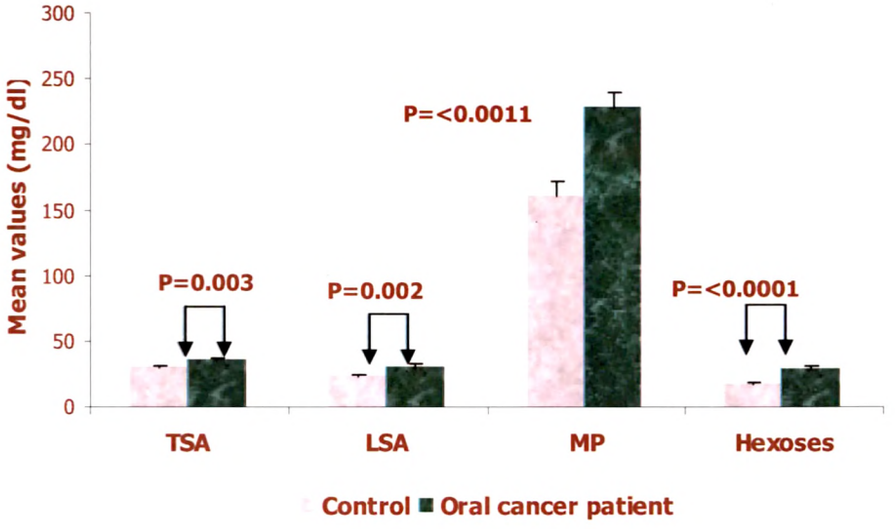


Table-4.2.47: Comparison of mean values of serum glycoprotein conjugates in subjects

Biomarkers	Serum glycoprotein conjugates (Mean± S.E.M)		'p' value
	Controls	Oral cancer patients	
TSA	29.3042±1.5898	35.4496-1.1450	**0.003**
LSA	22.9095±1.2727	30.4299±1.9457	**0.002**
MP	160.0850±11.8181	229.0143±11.9021	**<0.0001**
Hexoses	17.0650±1.1293	29.0782±1.5163	**<0.0001**

Receiver's Operating Characteristic Curve analysis

Receiver's Operating Curve were plotted and the ROC analysis revealed that all the glycoprotein conjugates including serum TSA, LSA, MP and Hexoses have significant efficacy to discriminate between controls and oral cancer patients. The area under the curve for the ROC analysis plotted for biomarker discrimination among subjects is shown with 95% confidence interval (CI) in **Figure-4.2.36** and statistical significance of the test is tabulated in **Table-4.2.48.**

Figure-4.2.36: ROC curve for serum glycoprotein constituents in controls vs oral cancer patients

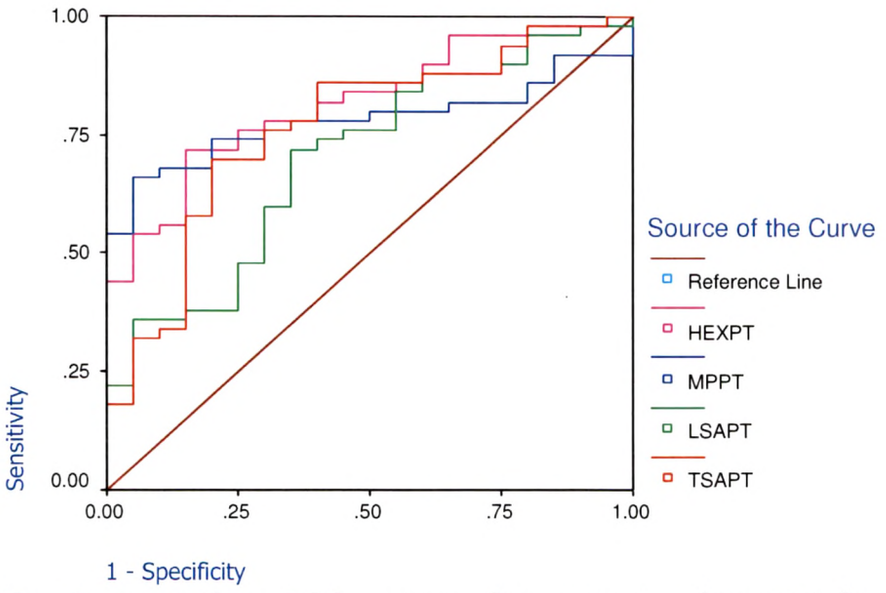


Table-4.2.48: Area under ROC curve for serum glycoprotein constituents in subjects (controls vs oral cancer patients)

Biomarkers	Area under the curve	'p' value	95% CI	
			Lower bound	Upper bound
TSA	0.766	**0.001**	0.643	0.889
LSA	0.701	**0.009**	0.569	0.833
MP	0.781	**0.000**	0.676	0.886
Hexoses	0.817	**0.000**	0.718	0.916

Comparison of serum glycoprotein constituents in the subjects associated with tobacco habit

Association of tobacco habits and mean values of serum glycoprotein conjugates was also evaluated in controls and oral cancer patients. In tobacco users (WHT), the mean levels of TSA (p=0.075), LSA (p=0.108), MP (p=0.028) and hexoses (p=<0.0001) were higher in oral cancer patients as compared to controls, as illustrated from **Table-4.2.49**. In non-tobacco users (NHT), the mean values of TSA (p=0.822), LSA (p=0.027), MP (p=0.336) and hexoses (p=0.052) were higher in oral cancer patients as compared to controls. In cancer patients, however the mean levels of TSA (0.127), MP (p=0.220), Hexoses (p=0.196) were also higher in tobacco users (WHT) as

compared to non-tobacco users (NHT). The mean levels of LSA (p=0.736) was lower in tobacco users as compared to non-tobacco users.

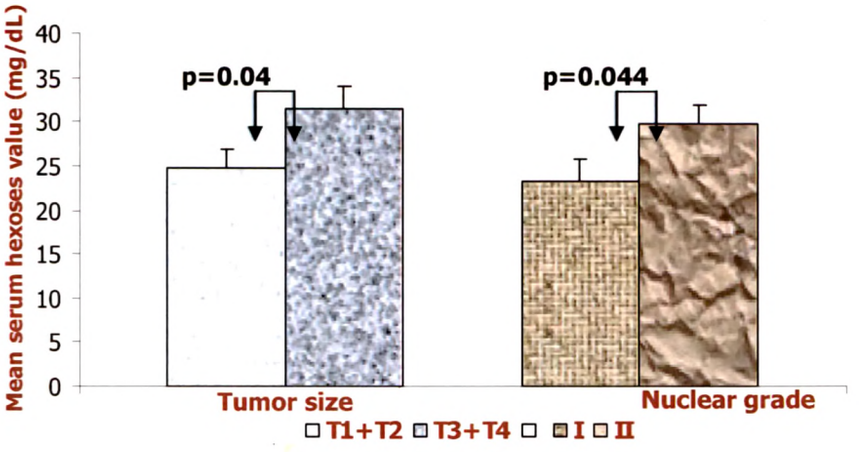
Association of serum glycoprotein constituents with clinico-pathological parameters in oral cancer patients by Univariate analysis

Serum glycoprotein constituents was also correlated with the clinico-pathological parameters like age, sex, tumor size and differentiation, nuclear grade, lymph node involvement, lymphatic response, tumor infilteration and stage of the disease of the oral cancer patients by Unpaired student's *t*-test analysis. As illustrated from **figure-4.2.37,** mean levels of serum hexoses were significantly higher in oral cancer patients with larger tumor size (p=0.04) and nuclear grade II (p=0.044) than smaller sized tumors and tumors with nuclear grade I.

Table-4.2.49: Mean values of serum glycoprotein constituents among tobacco user and nonusers of the subjects

Serum glycoprotein constituents	**Controls**	**Oral cancer patients**	**'p' value**
Tobacco habit (WHT)	**(Mean± S.E.M)**		
TSA	31.0036±2.1121	35.8411±1.2651	0.075
LSA	24.4143±2.7145	30.4568±2.2978	0.108
MP	159.4714±25.0861	232.5472±13.4070	**0.028**
Hexoses	15.1714±1.5208	29.6458±1.7137	**<0.0001**
No habit of tobacco (NHT)			
TSA	29.8841±2.5036	30.7486±2.8277	0.822
LSA	24.1000±1.1603	31.5756±2.2991	**0.027**
MP	160.60±15.5595	192.5401±27.8799	0.336
Hexoses	18.0091±1.6732	25.1118±2.8721	**0.052**
Oral cancer patients	**Tobacco habit (WHT)**	**No habit of tobacco (NHT)**	
TSA	35.8411±1.2651	30.7486±2.8277	0.127
LSA	30.4568±2.2978	31.5756±2.2991	0.736
MP	232.5472±13.4070	192.5401±27.8799	0.220
Hexoses	29.6458±1.7137	25.1118±2.8721	0.196

Figure-4.2.37: Comparison of mean values of serum hexoses in oral cancer patients with tumor size and nuclear grade



Further the association of these clinico-pathological parameters was determined using Univariate analysis **(Table-4.2.50).** Serum TSA, LSA, MP and hexoses were higher in patients with advanced stage as compared to patients with early stage of the disease. Serum LSA (p=0.024) was significantly associated with tumor infilteration. Serum MP (p=0.029) was associated significantly with stage of disease of oral cancer patients. While serum hexoses was significantly associated with nuclear grade (p=0.034) and tumor size (p=0.034) of oral cancer patients. The serum glycoprotein constituents did not associate significantly with other clinico-pathological parameters.

Association of serum glycoprotein constituents with the course of disease (Untreated levels and follow-up levels)

The association of serum glycoprotein constituents with the disease course was also studied by comparing the untreated levels with the follow-ups. **Table-4.2.51** shows the comparison of mean values of serum glycoprotein constituents between untreated oral cancer patients (PT) and their complete responders (CR) estimated by paired student's *t*-test analysis. No significant change was observed in the mean levels of serum TSA between untreated oral cancer and follow-up patients.

Table-4.2.50. Univariate analyses for association of clinico-pathological parameters with the presence of serum glycoprotein constituents

Clinico-pathological parameters	TSA		LSA		MP		Hexoses	
	F value	'p' value	F value	'p' Value	F value	'p' value	F value	'p' value
Age	0.046	0.830	0.121	0.730	0.159	0.691	2.254	0.137
Sex	3.248	0.075	0.908	0.345	1.400	0.240	0.075	0.786
Habit	1.746	0.190	0.027	0.869	0.967	0.328	0.773	0.382
Tumor differentiation	0.868	0.355	1.568	0.219	0.639	0.427	2.587	0.113
Nuclear Grade	0.715	0.401	1.105	0.301	0.198	0.658	4.913	**0.034**
Lymph-node metastasis	0.027	0.870	0.739	0.397	1.193	0.279	0.125	0.724
Tumor size	0.008	0.927	0.047	0.829	0.239	0.627	4.315	**0.04**
Stage of the disease	0.444	0.722	0.267	0.849	3.196	**0.029**	0.989	0.403
Early/advanced stage	0.032	0.858	0.401	0.530	1.004	0.320	2.390	0.127
Lymphatic response	0.030	0.864	0.125	0.728	0.214	0.646	1.870	0.179
Tumor infilteration	0.056	0.815	5.616	**0.024**	0.051	0.822	0.403	0.528

The mean values of serum LSA was higher significantly in untreated oral cancer patients as compared to the serum levels of complete-responder (CR) patients ($p=0.001$). While the mean levels of serum MP and serum hexoses was significantly higher in untreated oral cancer patients as compared to complete-responders ($p<0.001$ and $p=0.031$). The mean levels of serum MP and hexoses were lower in untreated oral cancer patients as compared to their non-responders. The oral cancer patients were followed up and monitored for serum glycoprotein constituents' levels and their representative cases of complete-responders and non-responders and the results are shown in **Figure-4.2.38** and **Figure-4.2.39**. In case-A a representative for complete responder, the oral cancer patient is a 54 yr old chronic smoker male suffering from squamous cell carcinoma of lower alveolus diagnosed with stage pT1N2Mx showing tumor infilteration into stroma and muscles. The mean serum glycoprotein values for TSA, LSA, MP and hexoses were measured before and after anticancer treatment (Surgery and Radiotherapy). As shown in **Figure-4.2.38** the levels were higher at untreated stage, which declined gradually in the last follow-up correlating well with disease progression and clinical details like no evidence of disease (NED). Patient showed good response to anticancer treatment.

Table-4.2.51: Comparison of mean values of serum glycoprotein constituents between untreated (PT) and follow-up (CR and NR) of the oral cancer patients

Oral cancer patients	Serum TSA Mean± S.E.M	Trend	Paired student's *t*-test 'p' value
Untreated oral cancer patients (PT)	31.628±1.1517		
Complete responders (CR)	35.660±2.869	Lower	0.243
Untreated oral cancer patients (PT)	42.802±3.218		
Non-responders (NR)	39.476±3.221	Higher	0.513
	Serum LSA		
Untreated oral cancer patients (PT)	23.707±3.078		
Complete responders (CR)	53.055±4.582	Lower	**0.001**
	Serum MP		
Untreated oral cancer patients (PT)	237.247±14.229		
Complete responders (CR)	125.237±8.210	Higher	**<0.0001**
Untreated oral cancer patients (PT)	119.191±29.310	Lower	**<0.0001**
Non-responders (NR)	169.007±12.864		
	Serum Hexoses		
Untreated oral cancer patients (PT)	24.766±1.8-011	Higher	**0.031**
Complete responders (CR)	20.024±0.949		
Untreated oral cancer patients (PT)	20.761±5.202		
Non-responders (NR)	28.562±3.468	Lower	0.207

Figure-4.2.38: Representative case for complete responder (CR)

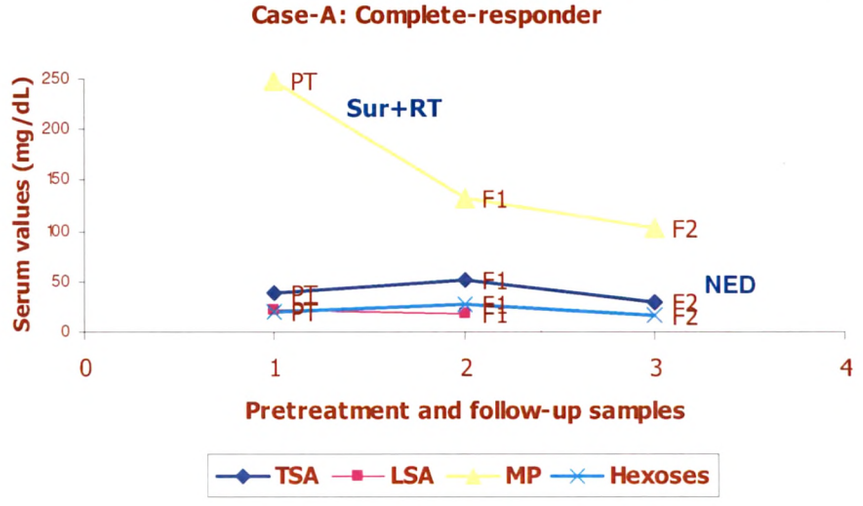
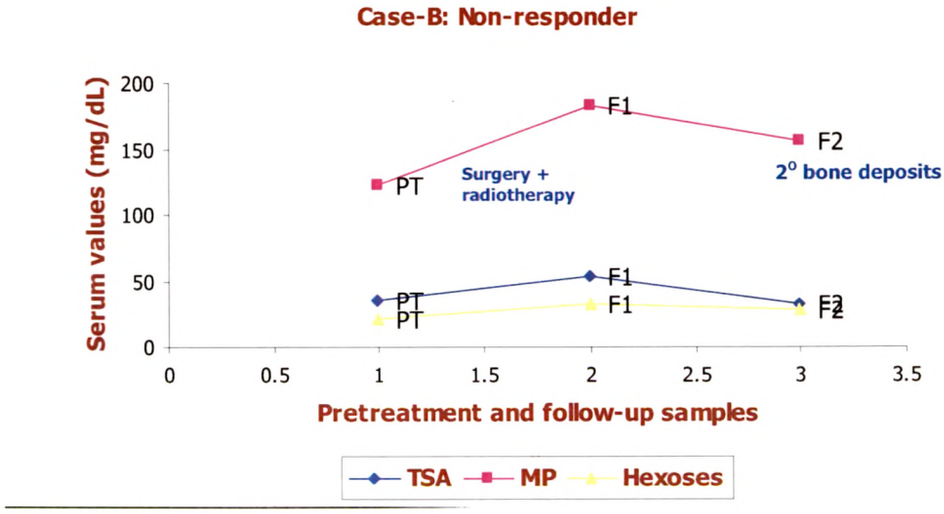


Figure-4.2.39 represents a case of non-responder who is a 42 yr old female patient suffering from squamous cell carcinoma of right tongue. The clinical

stage of the patient could not be assessed as anticancer therapy patient underwent surgery followed by radiotherapy and chemotherapy. The untreated serum levels for TSA, LSA, MP and hexoses were higher, which remained persistent with no changes during follow-up with complaint of pain and positive nodal status showing no response to therapy. The increased levels of the glycoprotein parameters correlated well with the disease status.

Figure-4.2.39: Representative case for non-responder (NR)



PT=Untreated sample; F1= first follow-up sample; F2=second follow-up sample

4.2.4 Circulating levels of Gelatinases and its natural inhibitors

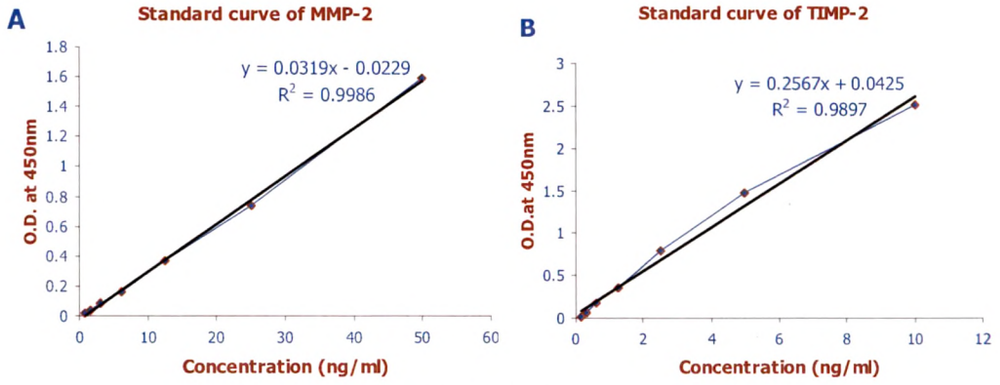
Tissue expression of gelatinases is known to relate to cancer progression and survival [Nelson et al., 2000], but more information is still needed regarding the clinical significance of circulating metalloproteinase in cancer and their validation as potential novel new markers in decision-making of clinicians. Action of MMPs is regulated and inhibited by their natural inhibitors termed as tissue inhibitors of matrix metalloproteinase (TIMPs). However, in addition to the tumor inhibiting function of TIMPs, opposing actions of these enzymes include apoptotic inhibition, angiogenic regulation and stimulation of cell growth. Degradation of extracellular matrix (ECM) is prerequisite in the process of angiogenesis, invasion and metastasis. Disruption of homeostasis is modulated by an imbalance between levels of these MMPs and TIMPs. Measurement of different components of MMP/TIMP system in circulation would be substantial to elucidate their role as tumor marker for clinical use in the management of these cancer patients. Therefore in the present study, plasma levels of both gelatinase-A (MMP-2) and gelatinase-B (MMP-9) and their inhibitors TIMP-1, and TIMP-2 were measured in 100 controls (healthy individuals), untreated oral cancer patients (PT) and their follow-ups to evaluate their clinical utility. Sandwich ELISA was used for analysis of plasma levels of all four proteins and commercially available kits were procured from R & D Systems. For every assay and batch, respective standards were run and curves were plotted, which were used for calculating the unknown levels of plasma MMPs and TIMPs.

Expression of plasma Gelatinase-A (MMP-2) and its Tissue Inhibitor TIMP-2 in oral cancer.

Gelatinase-A (MMP-2) and its inhibitor TIMP-2 bind to each other preferentially in 1:1 non-covalent interaction. These parameters were measured from plasma samples of subjects including controls and oral cancer patients. The oral cancer patients were further followed up during the course of the disease. Sandwich ELISA kits were used for estimating the plasma

levels of MMP-2 and TIMP-2 by using the respective standard curves as shown in **Figure-4.2.40 (A and B).**

Figure-4.2.40: Standard curve for (A) Plasma Gelatinase-A (MMP-2) and its (B) tissue inhibitor TIMP-2



Comparison of mean values of plasma Gelatinase-A (MMP-2), its inhibitor (TIMP-2) and MMP-2/TIMP-2 ratio in subjects.

Unpaired student's *t*-test was performed to compare the mean ± S.E.M values of gelatinase and its inhibitor among the subjects respectively. No significant change was seen in difference of MMP-2, TIMP-2, MMP-2/TIMP-2 and TIMP-2/MMP-2 in plasma levels of oral cancer patients and controls as illustrated from **Table-4.2.52**.

Table-4.2.52: Comparison of mean values of plasma MMP-2, TIMP-2, MMP-2/TIMP-2 and TIMP-2/MMP-2 in subjects

Biomarkers	Controls	Oral cancer patients	'p' value
	Mean ± S.E.M (ng/ml)		
MMP-2	195.8223±19.165	167.1357±8.911	0.192
TIMP-2	38.5508±1.783	35.0433±1.399	0.134
MMP-2/TIMP-2	5.12061±0.524	4.8228±0.227	0.609
TIMP-2/MMP-2	0.2425±0.0477	0.2178±0.010	0.621

Receiver's Operating Characteristic Curve analysis: Receiver's Operating Curves (ROC) were plotted to determine the discriminatory efficacy of plasma levels of gelatinase-A and its inhibitor TIMP-2 between both groups

of healthy controls and oral cancer patients **(Figure-4.2.41 and Table-4.2.53).** ROC analysis of the data suggests that the plasma levels of MMP-2, TIMP-2 and MMP-2/TIMP-2 index did not have good efficacy to discriminate between controls and oral cancer patients, but TIMP-2/MMP-2 ratio had good efficacy to discriminate between the two groups of subjects.

Figure-4.2.41: ROC curve for plasma Gelatinase - A and its Tissue Inhibitor in controls vs oral cancer patients

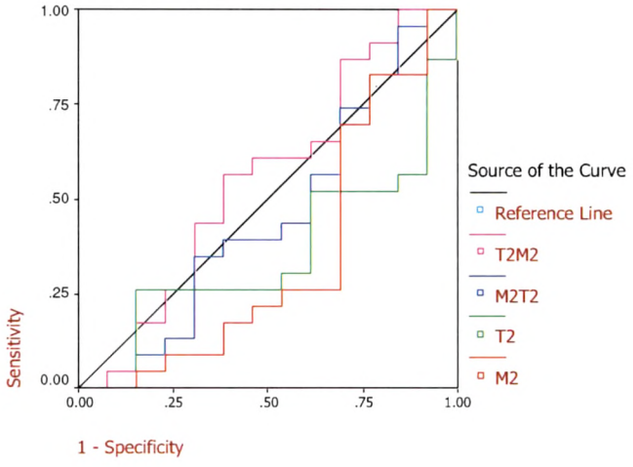


Table-4.2.53: Area under ROC curve for plasma Gelatinase – A and its Tissue Inhibitor in subjects (controls vs oral cancer patients)

Biomarker	Area under the curve	'p' value	95% CI	
			Lower bound	Upper bound
MMP-2	0.344	0.126	0.138	0.551
TIMP-2	0.355	0.152	0.170	0.539
MMP-2/TIMP-2	0.452	0.633	0.242	0.661
TIMP-2/MMP-2	0.548	0.633	0.339	0.758

Comparison of MMP-2 and TIMP-2 in the subjects associated with tobacco habit

The mean values of MMP-2, TIMP-2, and ratios of MMP-2/TIMP-2 and TIMP-2/MMP-2 were compared between subjects having habit of tobacco (WHT), those with no habit of tobacco (NHT) and also among the oral cancer patients with tobacco history. As shown in the **Table-4.2.54**, in case of oral cancer

patients, the mean of MMP-2/TIMP-2 ratio was significantly higher (p=0.008) in WHT as compared to NHT, whilst no significant change was observed for MMP-2, TIMP-2 and TIMP-2/MMP-2 ratio. In case of subjects with NHT, MMP-2 (p=0.005) and MMP-2/TIMP-2 (p=0.026) were significantly lower in oral cancer patients as compared to controls, whilst no significant change was observed for TIMP-2 and TIMP-2/MMP-2 ratio. However, no significant change was observed in mean plasma levels of MMP-2, TIMP-2, MMP-2/TIMP-2 and TIMP-2/MMP-2 ratio in case of subjects with WHT.

Table-4.2.54: Comparison of mean values of plasma Gelatinase-A, TIMP-1, MMP-2/TIMP-2 and TIMP-2/MMP-2 among tobacco users and nonusers of the subjects

Biomarkers	Plasma levels (ng/ml) (Mean± S.E.M)		'p' value
	Controls	Oral cancer patients	
Tobacco habit (WHT)			
MMP-2	125.4000±45.5223	169.4248±9.6036	0.437
TIMP-2	35.9067±2.3766	34.9486±1.5342	0.752
MMP-2/TIMP-2	3.4076±1.1032	4.9037±0.2408	0.306
TIMP-2/MMP-2	0.4139±0.1888	0.2144±0.01114	0.401
No habit of tobacco (NHT)			
MMP-2	216.9490±16.8404	143.1000±9.6000	**0.005**
TIMP-2	39.3440±2.1980	36.0500±0.3900	0.172
MMP-2/TIMP-2	5.6345±0.5185	3.9728±0.3093	**0.026**
TIMP-2/MMP-2	0.1911±0.01735	0.2532±0.01971	0.100
Oral cancer patients	**Tobacco habit (WHT)**	**No habit of tobacco (NHT)**	
MMP-2	170.1760±10.0655	146.8667±6.7013	0.075
TIMP-2	34.5420±1.5553	38.3933±2.3541	0.243
MMP-2/TIMP-2	4.9695±.2435	3.8400±0.2212	**0.008**
TIMP-2/MMP-2	0.2125±0.01102	0.2600±0.01528	0.058

Association of MMP-2 and TIMP-2 with clinico-pathological parameters in oral cancer patients by multivariate analysis

The clinico-pathological parameters like age, sex, tumor size and differentiation, nuclear grade, lymph node involvement, lymphatic response, tumor infiltration and stage of the disease of the oral cancer patients were associated with the expression of MMP-2, TIMP-2 in plasma levels. The association of these clinico-pathological parameters was also determined

using multivariate analysis (**Table-4.2.55**). The plasma levels of MMP-2, TIMP-2 were higher in aged oral cancer patients. MMP-2 was higher while TIMP-2 was lower in patients with tobacco use. MMP-2 was lower, while TIMP-2 was higher in moderately differentiated tumors and in patients with lymph-node metastasis. Both MMP-2 and TIMP-2 levels were lower in advanced stage as compared to early stage tumors. The levels were also higher in NG-II tumors and in patients with lymphatic response, while lower in patients with tumor infiltration. However, the plasma levels of both MMP-2 and TIMP-2 and the indices did not correlate with none of the clinico-pathological parameters of the oral cancer patients.

Table-4.2.55: Association of Gelatinase-A (MMP-2) and its inhibitor TIMP-2 with clinico-pathological parameters in oral cancer patients by multivariate analyses.

Clinico-pathological parameters	MMP-2		TIMP-2		MMP2/TIMP-2		TIMP-2/MMP-2	
	F value	'p' value	F value	'p' value	F value	'p' value	F value	'p' value
Age	0.001	0.981	0.779	0.388	0.739	0.400	0.200	0.660
Sex	0.005	0.943	0.005	0.944	0.041	0.841	0.073	0.790
Habit	0.656	0.427	0.031	0.861	1.237	0.279	0.983	0.333
Tumor Differentiation	0.009	0.925	0.003	0.958	0.028	0.868	0.164	0.690
Nuclear Grade	0.004	0.953	0.021	0.887	0.006	0.941	0.107	0.748
Lymph-node metastasis	0.335	0.573	0.069	0.797	1.756	0.208	2.012	0.180
Tumor size	1.181	0.291	3.025	0.099	0.112	0.742	0.154	0.699
Stage of the disease	1.168	0.353	0.423	0.739	1.082	0.385	1.456	0.264
Early/ advanced stage	0.166	0.688	0.170	0.685	0.005	0.943	0.001	0.974
Lymphatic response	1.139	0.311	0.046	0.835	2.931	0.118	3.129	0.107
Tumor infiltration	0.231	0.637	0.135	0.718	0.042	0.840	0.153	0.701

Comparison of plasma levels of MMP-2, TIMP-1, MMP-2/TIMP-2 and TIMP-2/ MMP-2 untreated oral cancer patients and their follow-ups.

Table-4.2.56 shows the comparison of mean values of MMP-2, TIMP-2 and indices between untreated oral cancer patients (PT) and their complete responders (CR) and non-responders (NR). The mean values of MMP-2

(p=0.013) and TIMP-2 (p=0.049) significantly lower in PT as compared to their CR. While no significant change was observed in the mean levels of MMP-2 and TIMP-2 between PT and NR.

Table-4.2.56: Comparison of mean values of plasma MMP-2, and its inhibitor TIMP-2 between untreated (PT) and follow-up (CR and NR) of the oral cancer patients

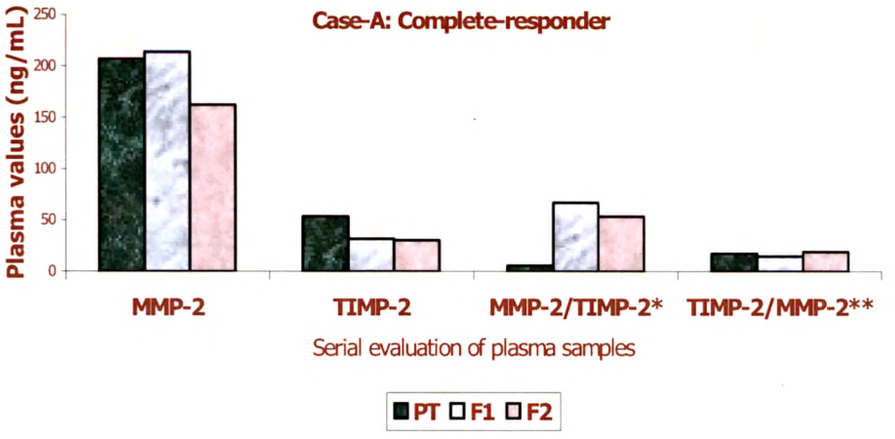
Oral cancer patients	Plasma MMP-2 Mean± S.E.M	Trend	'p' value
Untreated oral cancer patients (PT)	165.7719±9.012		
Complete responders (CR)	199.8850±11.487	Lower	**0.013**
Untreated oral cancer patients (PT)	154.900±9.350	Lower	
Non-responders (NR)	168.3838 ±12.736		0.235
	Plasma TIMP-2		
Untreated oral cancer patients (PT)	35.2000±1.391	Lower	**0.049**
Complete responders (CR)	38.7231±1.593		
Untreated oral cancer patients (PT)	35.1188±2.600	Higher	0.896
Non-responders (NR)	34.6463±2.350		

Association with the course of disease (Untreated levels and follow-up levels)

The association of plasma levels of Gelatinase-A (MMP-2) and its inhibitor TIMP-2 with the disease course was also studied by comparing the untreated levels with the follow-ups. The oral cancer patients were followed up and monitored for levels and their representative cases of complete-responders and non-responders are shown in **Figure-4.2.42** and **Figure-4.2.43.**

In case-A, a representative for complete responder, the oral cancer patient is a 54 yr old male chronic smoker suffering from squamous cell carcinoma of lower alveolus diagnosed with stage pT1N2Mx showing tumor infiltration into stroma and muscles. The mean plasma MMP-2, TIMP-2 values were measured before and after anticancer treatment. As shown in **Figure-4.2.42**, the levels were higher at untreated stage, which declined gradually in the last follow-up correlating well with disease progression and clinical outcome of patients, like no evidence of disease (NED). Patient showed good response to anticancer treatment.

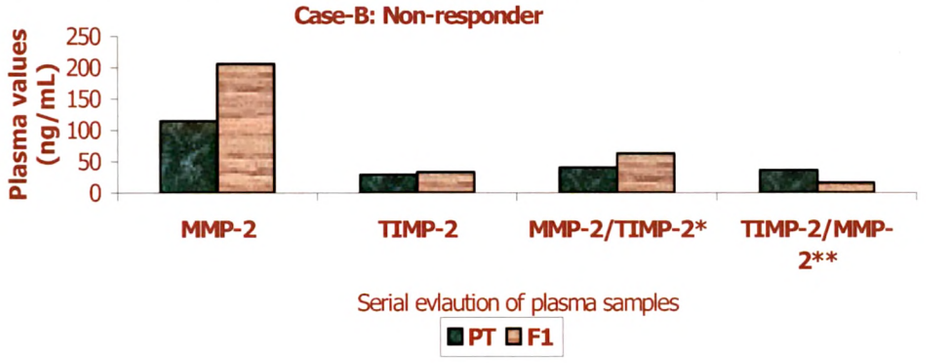
Figure-4.2.42: Representative case for complete responder (CR)



PT=Untreated sample; F1= first follow-up sample; F2=second follow-up samples

In case-B, the patient representing a non-responder is 30 yr old chronic male where the levels of MMP-2, TIMP-2 and MMP-2/TIMP-2 ratio of tobacco user, diagnosed with squamous cell carcinoma of buccal mucosa PT4N2bM0, stage IV. The patient was followed-up, but was then discharged as not responding to treatment and was sent only for palliative treatment of pain. The plasma levels of MMP-2 and TIMP-2 MMP-2/TIMP-2 ratio were found to be higher than the untreated levels correlating well again with the clinical response of patient as depicted in the **Figure–4.2.43**.

Figure-4.2.43 Representative case for Non-responder (NR)

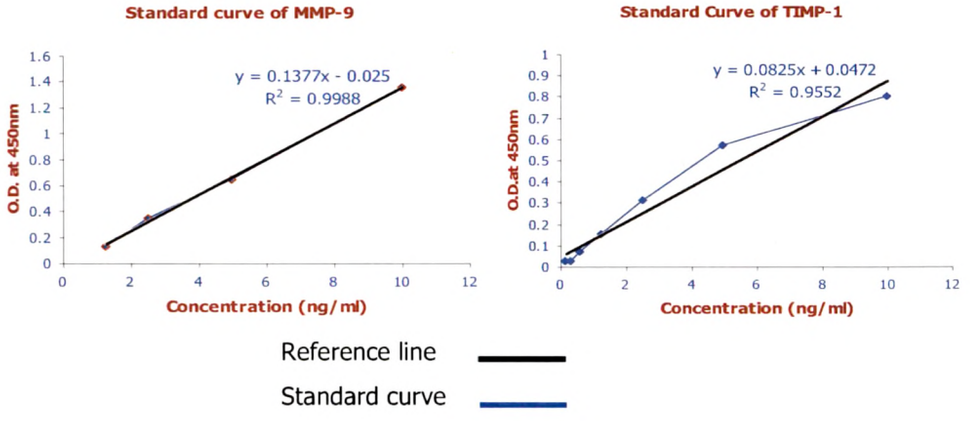


PT=Untreated sample; F1= first follow-up sample; F2=second follow-up samples

Expression of plasma Gelatinase-B (MMP-9) and its Tissue Inhibitor TIMP-1 in oral cancer.

Gelatinase-B (MMP-9) and tissue inhibitor TIMP-1 bind to each other preferentially in 1:1 non-covalent interaction. These parameters were measured form plasma samples of subjects including controls and oral cancer patients. These oral cancer patients were further followed up during the course of disease and blood samples were also taken from them to evaluate their plasma levels. Sandwich ELISA kits were used for estimating the plasma levels of MMP-9 and TIMP-1. For each batch of assay, standards of MMP-9 and TIMP-1 were used to plot a standard curves as shown in **Figure-4.2.44 A and B respectively.**

Figure-4.2.44: Standard curve for (A) Gelatinase-B (MMP-9) standard and (B) Standard curve for TIMP-1 standard



Comparison of mean values of plasma Gelatinase-B (MMP-9), its inhibitor (TIMP-1) and MMP-9/TIMP-1 ratio in subjects:

Table-4.2.57 shows the unpaired analysis for comparison of mean plasma levels of Gelatinase and its tissue inhibitor measured among the subjects respectively. Unpaired student's *t*-test analysis revealed that the mean values of MMP-9 (p=0.886), TIMP-1 (p=0.286) and MMP-9/TIMP-1 ratio (p=0.426) were lower and TIMP-1/MMP-9 ratio (p=0.446) was higher in plasma of oral cancer patients than the healthy controls. Nevertheless, no significant change was observed.

Table-4.2.57: Comparison of mean values of plasma MMP-9, TIMP-1, MMP-9/TIMP-1 and TIMP-1/MMP-9 in subjects

Biomarkers	Controls	Oral cancer patients	'p' value
	Mean ± S.E.M (ng/ml)		
MMP-9	541.186±82.425	526.802±55.854	0.886
TIMP-1	65.343±7.691	55.918±3.77	0.286
MMP-9/TIMP-1	21.190±13.609	9.941±1.160	0.426
TIMP-1/MMP-9	0.1524±0.0257	0.230±0.0978	0.446

Receiver's Operating Characteristic Curve analysis

Receiver's Operating Curve (ROC) were plotted to determine the discriminatory efficacy of plasma levels of Gelatinase-B and its inhibitor TIMP-1 in both groups of healthy controls and oral cancer patients. The analysis suggests the plasma levels of MMP-9, TIMP-1, and ratio of TIMP-1/MMP-9 did not have good efficacy to discriminate between controls and oral cancer patients. However, MMP-9/TIMP-1 ratio had good efficacy to discriminate between the two groups of subjects. The area under the curve for the ROC analysis plotted for biomarker discriminating among subjects is shown in **Figure-4.2.45** while 95% confidence interval (CI) and statistical significance of the test is tabulated in **Table-4.2.58.**

Figure-4.2.45: ROC curve for plasma Gelatinase- B and its Tissue Inhibitor in controls vs oral cancer patients

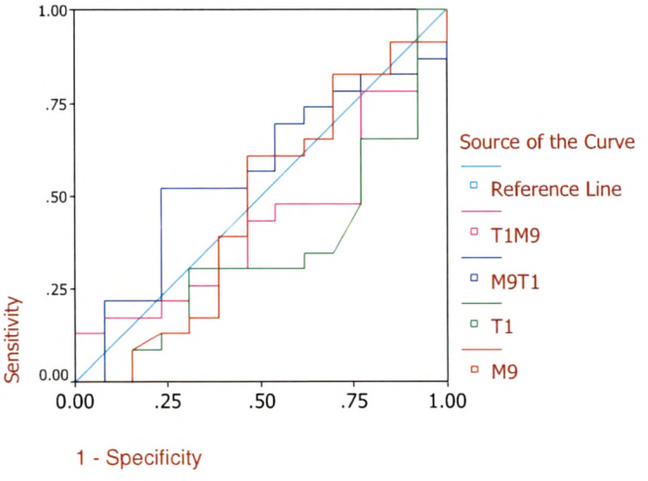


Table-4.2.58: Area under ROC curve for plasma Gelatinase - B and its Tissue Inhibitor in subjects (controls vs oral cancer patients)

Biomarker	Area under the curve	'p' value	95% CI	
			Lower bound	Upper bound
MMP-9	0.473	0.792	0.262	0.685
TIMP-1	0.346	0.130	0.155	0.537
MMP-9/TIMP-1	0.562	0.542	0.366	0.758
TIMP-1/MMP-9	0.438	0.542	0.242	0.634

Comparison of plasma MMP-9 and TIMP-1 in the subjects associated with tobacco habit of subjects

The circulating levels of plasma gelatinases and its inhibitor were associated with the tobacco habits of oral cancer patients and controls. The mean values of MMP-9, TIMP-1, and ratios of MMP-9/TIMP-1 TIMP-1/MMP-9 were compared between subjects having habit of tobacco (WHT), those with no habit of tobacco (NHT) and also among the oral cancer patients.

Table-4.2.59: Comparison of mean values of plasma Gelatinase-B, TIMP-1, MMP-9/TIMP-1 and TIMP-1/MMP-9 between tobacco users and nonusers of the subjects

Biomarkers	Plasma levels (ng/ml) (Mean± S.E.M)		'p' value
	Controls	Oral cancer patients	
Tobacco habit (WHT)			
MMP-9	622.6667±218.4267	525.1457±59.9736	0.704
TIMP-1	89.8633±15.6855	56.7524±4.0071	0.162
MMP-9/TIMP-1	6.4864±1.8519	9.8143±1.2682	0.209
TIMP-1/MMP-9	0.1930±0.06868	0.2441±0.1069	0.695
No habit of tobacco (NHT)			
MMP-9	516.7420±91.1466	544.2000±183.4000	0.909
TIMP-1	57.9880±7.7522	47.1600±11.8900	0.526
MMP-9/TIMP-1	25.6021±17.6482	11.2757±1.0461	0.438
TIMP-1/MMP-9	0.1402±0.0274	0.08946±0.08299	0.107
Oral cancer patients	**Tobacco habit (WHT)**	**No habit of tobacco (NHT)**	
	(Mean± S.E.M)		
MMP-9	517.1930±62.4943	590.8667±115.713	0.611
TIMP-1	57.8925±4.0386	42.7567±8.155	0.192
MMP-9/TIMP-1	9.2970±1.2176	14.2333±3.0192	0.237
TIMP-1/MMP-9	0.2545±.01117	0.07667±0.1453	0.130

As illustrated from **Table-4.2.59**, in case of subjects with tobacco habits (WHT), the mean plasma levels of MMP-9 (p=0.704) and TIMP-1 (p=0.162)

were lower, while MMP-9/TIMP-1 (p=0.209) and TIMP-1/MMP-9 (p=0.695) indices were higher in oral cancer patients as compared to healthy controls. In case of subjects with no habit of tobacco, (NHT), the mean plasma levels of MMP-9 (p=0.909) were higher, while TIMP-9 (p=0.526), MMP-9/TIMP-1 (p=0.438) and TIMP-1/MMP-9 (p=0.107) were higher in healthy controls as compared to oral cancer patients. In case of oral cancer patients, MMP-9 (p=0.611) and MMP-9/TIMP-1 (p=0.237) was lower, while TIMP-1 (p=0.192) and TIMP-1/ MMP-9 (p=0.130) index was higher in oral cancer patients with WHT as compared to NHT. However, among all these subjects, no significant change was seen in the plasma levels of MMP-9 an TIMP-1 with tobacco habits.

Association of MMP-9 and TIMP-1 with clinico-pathological parameters in oral cancer patients by multivariate analysis

The plasma levels of MMP-9 and TIMP-1 were correlated with the clinico-pathological parameters like age, sex, tumor size and differentiation, nuclear grade, lymph node involvement, lymphatic response, tumor infiltration and stage of the disease of the oral cancer patients. Further the association of plasma levels of Gelatinase-B and its inhibitor TIMP-1 with the clinico-pathological parameters was determined using multivariate analysis (**Table-4.2.60**).

The plasma levels of MMP-9 and TIMP-1 were lower in aged and higher in male patients. MMP-9 was lower while TIMP-1 was higher in patients with tobacco habit. Both MMP-9 and TIMP-1 were higher in moderately differentiated and NG-II and large sized tumors. MMP-9 was lower, while TIMP-1 was higher in patients with lymph-node metastasis as compared to those without lymph-node metastasis. The plasma levels were also higher in advanced stage of patients as compared to early stage patients. MMP-9 was lower in patients with lymphatic response and tumor infiltration. TIMP-1 levels were higher in patients with lymphatic response, and lower in patients with tumor infiltration. The plasma levels of MMP-9, TIMP-1 and the indices did not

correlate with none of the clinico-pathological parameters of the oral cancer patients.

Table-4.2.60: Association of Gelatinase-B (MMP-9) with clinico-pathological parameters in oral cancer patients by multivariate analysis.

Clinico-pathological parameters	MMP-9		TIMP-1		MMP9/TIMP-1		TIMP-1/MMP-9	
	F value	'p' value	F value	'p' value	F value	'p' value	F value	'p' value
Age	0.149	0.704	1.061	0.315	0.325	0.575	0.310	0.584
Sex	0.184	0.673	0.026	0.874	0.014	0.908	0.286	0.599
Habit	0.003	0.956	0.507	0.485	0.097	0.759	0.195	0.664
Tumor differentiation	0.007	0.932	0.000	0.995	0.028	0.870	0.021	0.886
Nuclear grade	0.131	0.722	0.407	0.532	0.025	0.877	0.000	0.999
Lymph-node metastasis	1.389	0.260	0.768	0.397	1.739	0.210	0.317	0.583
Tumor size	0.812	0.379	4.249	0.054	0.158	0.696	0.687	0.418
Stage of the disease	0.130	0.941	0.810	0.507	0.049	0.985	1.231	0.331
Early/advanced stage	0.368	0.552	2.286	0.148	0.106	0.749	2.025	0.172
Lymphatic response	0.021	0.887	0.027	0.873	0.326	0.580	0.101	0.758
Tumor infiltration	0.511	0.484	0.681	0.421	0.051	0.825	0.237	0.632

Comparison of plasma MMP-9, and its inhibitor TIMP-1 between untreated (PT) and follow-up of the oral cancer patients

Table-4.2.61 shows the comparison of mean values of plasma levels of MMP-9 and TIMP-1 between untreated oral cancer patients (PT) and their respective complete responders (CR) and non-responders (NR) by paired student t-test analysis. The mean values of plasma MMP-9 (p=0.024) and TIMP-1 (p=0.013) were significantly lower in untreated oral cancer patients as compared to their complete responders. While the mean values of plasma MMP-9 (p=0.724) was higher and TIMP-1 (p=0.536) was lower in untreated oral cancer patients as compared to non-responders.

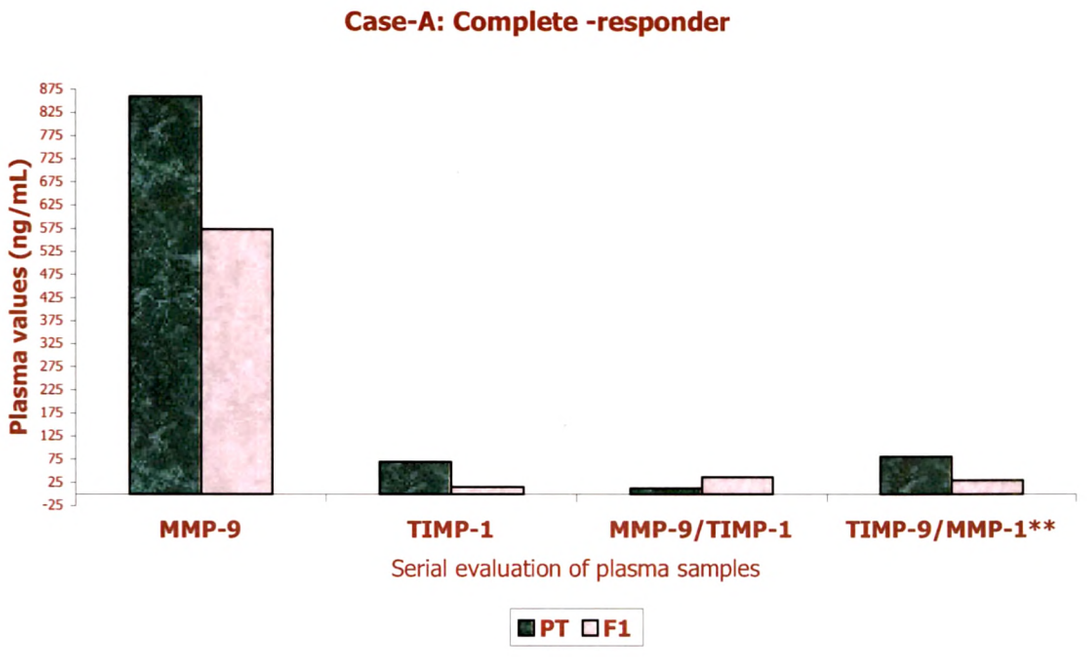
Table-4.2.61: Comparison of mean values of plasma MMP-9, and its inhibitor TIMP-1 between untreated (PT) and follow-up (CR and NR) of the oral cancer patients

Oral cancer patients	Plasma MMP-9 Mean± S.E.M	Trend	'p' value
Untreated oral cancer patients (PT)	396.4923±47.1413	Lower	**0.024**
Complete responders (CR)	537.6454±68.1290		
Untreated oral cancer patients (PT)	516.3900±77.3545	Higher	0.724
Non-responders (NR)	453.2300±120.233		
	Plasma TIMP-1		
Untreated oral cancer patients (PT)	49.6985±2.8976	Lower	**0.013**
Complete responders (CR)	63.6865±5.7337		
Untreated oral cancer patients (PT)	51.1263±8.111	Lower	0.537
Non-responders (NR)	59.1075±12.6630		

Association of with the course of disease (Untreated levels and follow-up levels)

The association of plasma levels of Gelatinase-A (MMP-2) and its inhibitor TIMP-2 with the disease course was also studied by comparing the untreated levels with the follow-ups. The oral cancer patients were followed up and monitored for levels and their representative cases of complete-responders and non-responders are shown in **Figure-4.2.46** and **Figure-4.2.47**. In case-A, the oral cancer patient is a 25 yr old male, chronic alcoholic and tobacco user (chewing and snuffing) suffering from squamous cell carcinoma of right buccal mucosa diagnosed at stage IV. Plasma values for MMP-9 and TIMP-1 were higher at the untreated stage. The patient received Surgery and Radiotherapy treatment and during follow-up, plasma levels of both MMP-9 and TIMP-1 were declined, which correlated well with clinical details, showing good response to treatment as the patient was presented with locally no evidence of disease (NED).

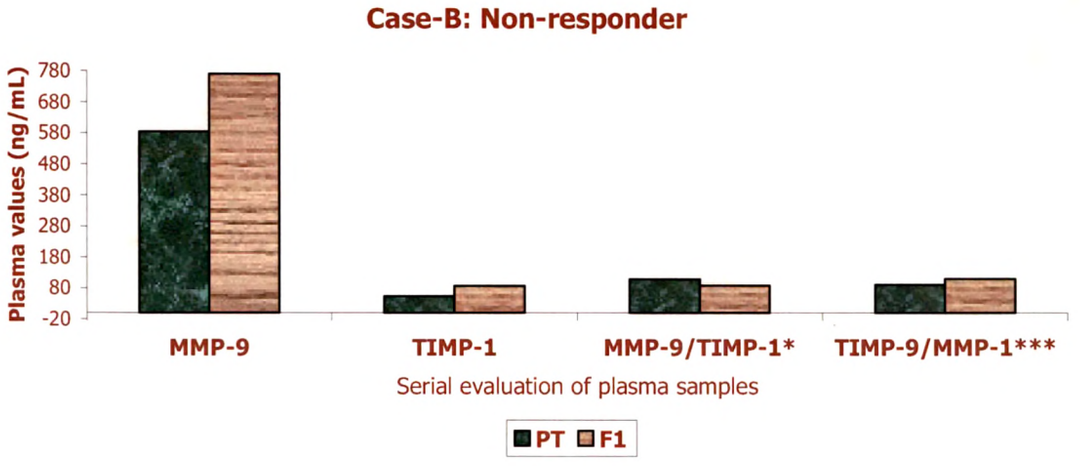
Figure-4.2.46: Representative case for complete responder (CR)



PT=Untreated sample; F1= first follow-up sample; F2=second follow-up samples

Case-B represents a non responder oral cancer patient who is a 54 yr old male, chronic smoker suffering from squamous cell carcinoma of lower alveolus diagnosed with stage pT1N2Mx showing tumor infiltration into stroma and muscles. The mean plasma MMP-2, TIMP-2 values were measured before and after anticancer treatment (Surgery and Radiotherapy). As shown in **Figure-4.2.47**, the levels were higher at untreated stage and also increased during the follow-up period. MMP-9/TIMP-1 was comparable, while TIMP-1/MMP-9 ratio was high during follow up. The patient when sent for palliative treatment showing poor response to therapy.

Figure-4.2.47: Representative case for Non-responder (NR)

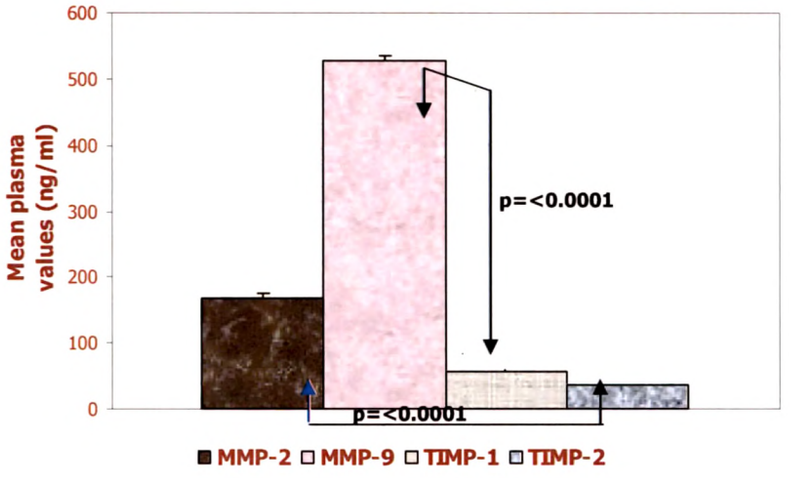


PT=Untreated sample; F1= first follow-up sample; F2=second follow-up samples

Comparison of Gelatinase-A (MMP-2), Gelatinase-B (MMP-9), tissue inhibitors TIMP-1 and TIMP-2, and MMP/TIMP indices in oral cancer patients

Both Gelatinases are regulated by their respective tissue inhibitors and comparing their respective levels gives us an opportunity to visualize picture of their expression in oral cancer. **Table-4.2.62** compares the plasma levels of MMP-2, MMP-9, TIMP-1, TIMP-2 and their ratios in oral cancer patients by paired student's *t*-test (**Figure-4.2.48**). Plasma levels of MMP-2 were positively correlated and were significantly lower in oral cancer patients as compared to MMP-9. Plasma levels of TIMP-1 were significantly higher as compared to plasma TIMP-2 in oral cancer patients with positive correlation. Plasma levels of MMP-2 and MMP-9 were significantly higher than both the natural tissue inhibitors, TIMP-1 and TIMP-2 in oral cancer patients.

Figure-4.2.48 Comparison of plasma levels of Gelatinase-A (MMP-2), Gelatinase-B (MMP-9), Tissue Inhibitors TIMP-1 and TIMP-2.



Comparison of indices revealed that MMP-2/TIMP-2 was significantly higher than TIMP-2/MMP-2 and lower than MMP-9/TIMP-1 respectively. MMP-9/TIMP-1 index was significantly higher than TIMP-1/MMP-9. However, TIMP-1/MMP-9 was significantly higher as compared to TIMP-2/MMP-2 in oral cancer patients.

Correlation of tissue and plasma levels of Gelatinase-A (MMP-2) and Gelatinase-B (MMP-9) in oral cancer patients

Expression of these enzymes in tissue and circulatory system were compared to evaluate their correlation in cancer. Spearmans rho bivariate coefficient of correlation was used to associate the levels of Gelatinases both total MMP-2 and total MMP-9 in tissue and plasma samples of the oral cancer patients. The results of correlation and its significance are summarized in the **Table-4.2.63**.

Table-4.2.62 Comparison of plasma levels of Gelatinase-A (MMP-2), Gelatinase-B (MMP-9) and their respective Tissue Inhibitors, TIMP-1, TIMP-2 with MMP/TIMP ratios in oral cancer patients

Oral cancer patients	Mean± S.E.M	Trend	'p' value	Correlation	'p' value
MMP-2	167.1357±8.911	Lower	**<0.0001**	Positive	0.602
MMP-9	526.8026±55.854				
TIMP-1	55.9183±3.770	Higher	**<0.0001**	Positive	0.950
TIMP-2	35.0443±1.399				
MMP-2/TIMP-2	4.8228±.227	Higher	**<0.0001**	Negative	**<0.001**
TIMP-2/MMP-2	0.2178±0.0104				
MMP-9/TIMP-1	9.9414±1.160	Higher	**<0.0001**	Negative	**0.011**
TIMP-1/MMP-9	0.2307±0.0978				
MMP-2/TIMP-2	4.8228± 0.227	Lower	**0.001**	Negative	0.111
MMP-9/TIMP-1	9.9414 ±1.160				
TIMP-2/MMP-2	0.2178±0.0104	Lower	0.899	Negative	0.317
TIMP-1/MMP-9	0.2307±0.0978				
MMP-2	167.1357±8.911	Higher	**<0.0001**	Positive	**0.004**
TIMP-2	35.0443±1.399				
MMP-9	526.8026±55.854	Higher	**<0.0001**	Positive	0.324
TIMP-1	55.9183±3.770				
MMP-2	167.1357±8.911	Higher	**<0.0001**	Positive	0.132
TIMP-1	55.9183±3.770				
MMP-9	526.8026±55.854	Higher	**<0.0001**	Positive	0.376
TIMP-2	35.0443±1.399				

Plasma MMP-2 correlated significantly positive with plasma levels of TIMP-2 and MMP-2/TIMP-2 index and significantly negative with TIMP-2/MMP-2 index. While plasma MMP-9 correlated positively with both TIMP-1 and MMP-9/TIMP-1 index and negatively with TIMP-1/MMP-9 index. Tissue Gelatinase-A (MMP-2) positively correlated with plasma levels of MMP-2, MMP-9, TIMP-1 and negatively correlated with TIMP-2. While tissue Gelatinase-B (MMP-9) correlated positively with plasma levels of MMP-9; TIMP-1 and TIMP-2 correlated negatively with plasma MMP-2 in oral cancer patients.

Table-4.2.63 Correlation of plasma values of Gelatinase-A (MMP-2), Gelatinase-B (MMP-9) with their respective Tissue Inhibitors, TIMP-1, TIMP-2 with MMP/TIMP ratios in oral cancer patients

Parameters	Spearmans Rho correlation	'p' value
MMP-2		
TIMP-2	0.483	**0.019**
MMP-2/TIMP-2	0.736	**<0.0001**
TIMP-2/MMP-2	-0.736	**<0.0001**
MMP-9		
TIMP-1	0.249	0.252
MMP-9/TIMP-1	0.831	**<0.0001**
TIMP-1/MMP-9	-0.831	**<0.0001**
Total MMP-2 (Tissue)		
Plasma MMP-2	0.300	0.624
Plasma MMP-9	0.600	0.285
Plasma TIMP-1	0.300	0.624
Plasma TIMP-2	-0.359	0.553
Total MMP-9 (Tissue)		
Plasma MMP-2	-0.200	0.747
Plasma MMP-9	0.100	0.873
Plasma TIMP-1	0.300	0.624
Plasma TIMP-2	0.410	0.493

4.2.5 Circulating levels of antioxidants and detoxifying enzymes

The antioxidants are known to reflect the redox state of cancerous cells. They are involved in detoxifying the lethal effects of ROS mediated oxidative damage incurred in cancer patients. Therefore, the plasma as well as erythrocyte levels of antioxidants such as Glutathione S-transferase (GST), Glutathione reductase (GR), Superoxide dismutase (SOD), Catalase and thiol were assessed in oral cancer patients by highly specific and sensitive spectrophotometric assays.

GST and GR levels were analysed from plasma and erythrocyte samples. Thiol levels were estimated from plasma. SOD and Catalase activity were estimated from erythrocyte samples. These biomarkers were also evaluated for clinical usefulness by comparing them with controls (healthy individuals), in samples of follow up oral cancer patients.

Figure-4.2.49 shows the comparison of levels of antioxidant and detoxification enzymes as well as thiol in controls and oral cancer patients.

Mean GST (from plasma and erythrocyte) and erythrocyte GR activities were significantly higher (p=0.042, p=0.0001 and p=0.0001, respectively) in cancer patients than healthy individuals. Mean levels of plasma GR and thiol as well as erythrocyte SOD were significantly lower (p=0.0001, p=0.0001 and p=0.001, respectively) in cancer patients than healthy individuals. Mean erythrocyte Catalase activity was also lower in cancer patients as compared to healthy individuals.

Receiver's Operating Characteristic Curve analysis

ROC curve analysis (**Figure-4.2.50 A**) revealed that plasma GR and thiol levels showed significantly higher accuracy in differentiating between healthy individuals and cancer patients. The area under curve for plasma GR and Thiol were 0.795 (p=0.0001) and 0.852 (p=0.0001), respectively. ROC curve analysis (**Figure-4.2.50 B**) revealed that erythrocyte GST, GR and Catalase levels also showed significantly higher accuracy in differentiating between healthy individuals and cancer patients. The area under curve for erythrocyte GST, GR and Catalase were 0.606 (p= 0.005), 0.850 (p=0.0001) and 0.608 (p=0.005), respectively. Erythrocyte SOD and plasma GST levels showed higher accuracy in differentiation between healthy individuals and cancer patients.

Figure-4.2.49: Comparison of antioxidant and detoxification enzymes as well as thiol levels between controls and cancer patients

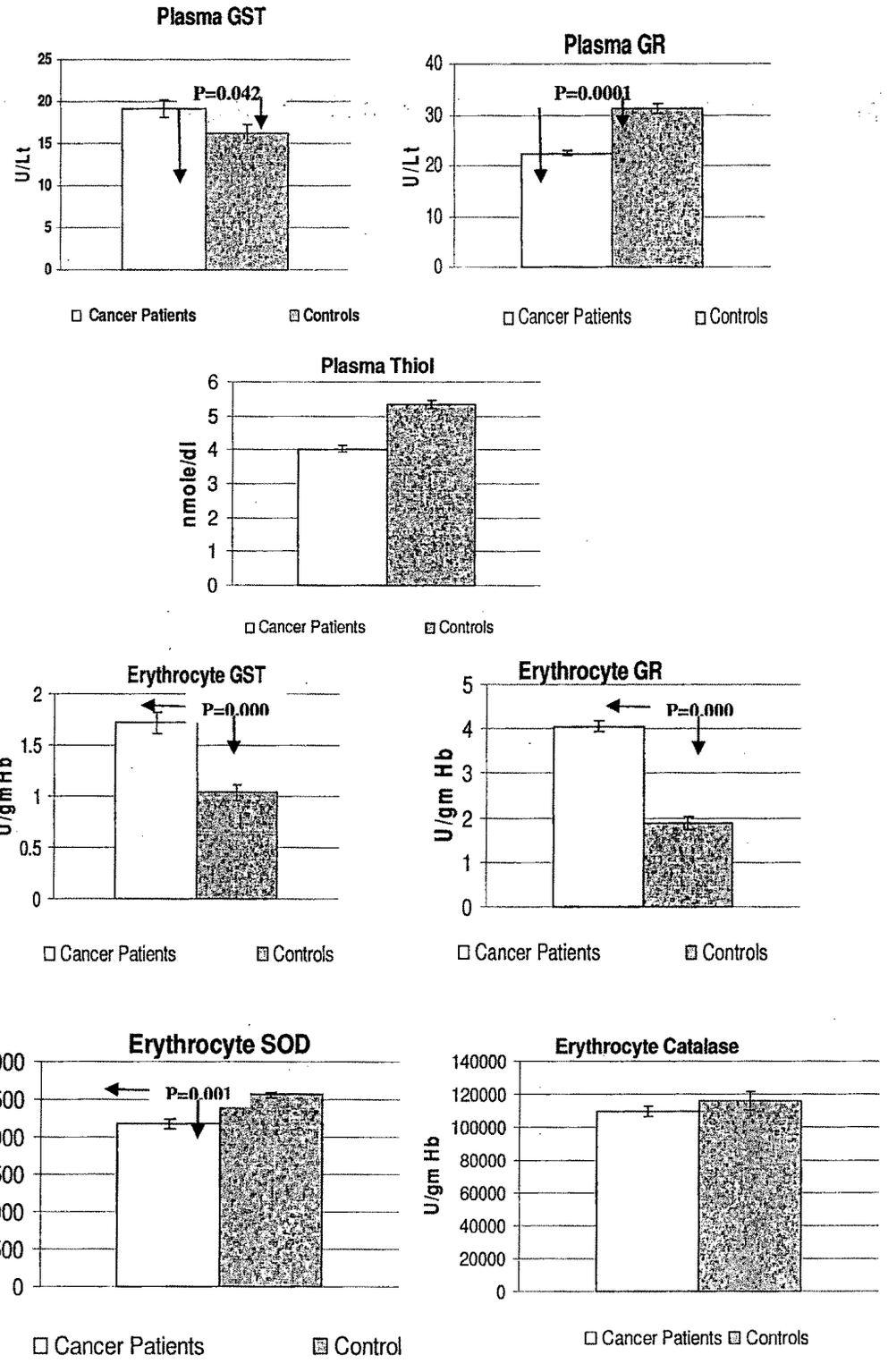


Figure-4.2.50 A & B: ROC curve for serum antioxidant and detoxifying enzymes in controls vs oral cancer patients

A B

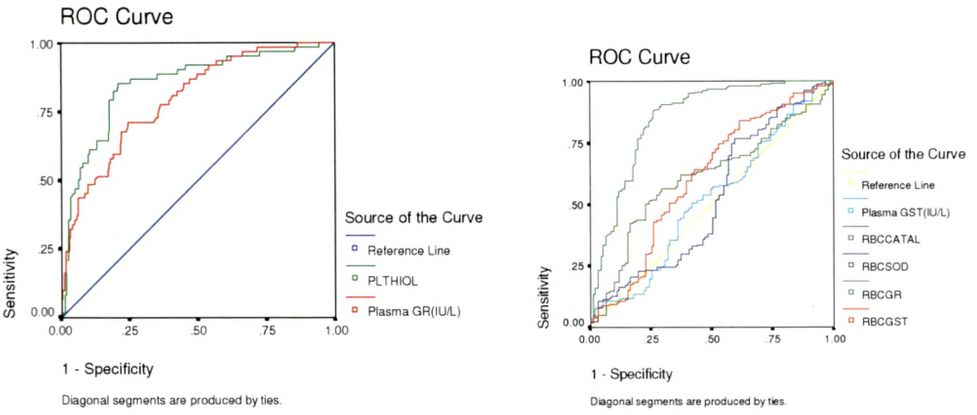


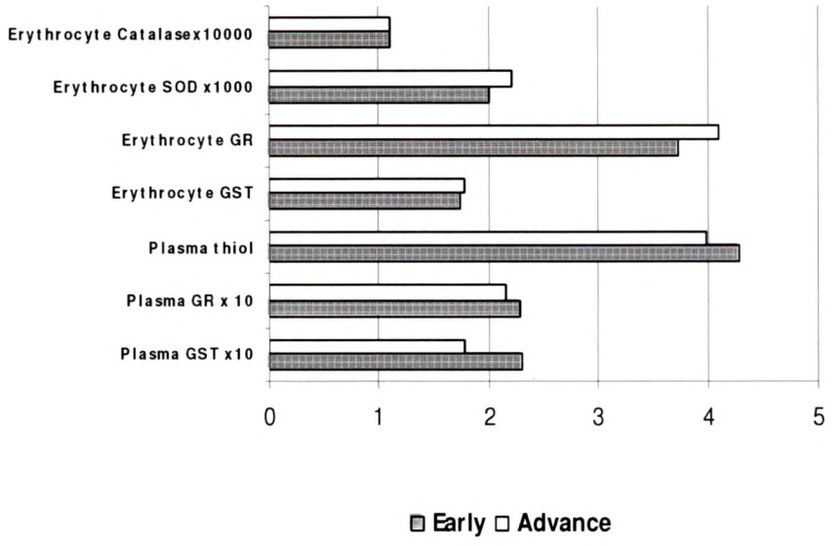
Table-4.2.64: Area under ROC curve for blood antioxidant and detoxifying enzymes in subjects (controls vs oral cancer patients)

Biomarkers	AUC	SE	'p' value	Lower bound	Upper bound
Plasma GR	0.795	0.032	**0.000**	0.732	0.858
Plasma Thiol	0.852	0.030	**0.000**	0.793	0.910
Erythrocyte GST	0.606	0.039	**0.005**	0.531	0.682
Erythrocyte GR	0.850	0.027	**0.000**	0.797	0.903
Erythrocyte SOD	0.522	0.040	0.565	0.444	0.600
Erythrocyte Catalase	0.608	0.037	**0.005**	0.536	0.679
Plasma GST	0.516	0.039	0.680	0.440	0.592

Correlation of the antioxidant and detoxifying enzymes with clinico-pathological parameters

All the circulating biomarkers involving antioxidants and detoxifying enzymes were compared between early (stage I+ II) and advanced (stage III + IV) stages in oral cancer patients. **Figure-4.2.51** shows comparison of mean levels of the biomarkers in early and advanced stages of cancer patients. Mean erythrocyte GR and SOD activities were higher in advanced stage of cancer patients than early disease. Mean values of plasma GST and plasma Thiol were lower in patients with advanced stage of cancer than the patients with early stage.

Figure-4.2.51: Correlation of antioxidant and detoxifying enzymes with the stage of the disease



Association of serum antioxidant and detoxifying enzymes with the course of disease (Untreated levels and follow-up levels)

Comparison of erythrocyte GST, GR, SOD and Catalase markers with treatment response is shown in **Figure-4.2.52**. Mean values of erythrocyte GST were significantly decreased in CR (p=0.002) and erythrocyte Catalase activities were also significantly decreased in NR (p=0.046) as compared to PT. Mean levels of erythrocyte catalase, SOD and GR were comparable between CR and PT, while levels of SOD, GR, GST were comparable between PT and NR.

Figure-4.2.52 Mean erythrocyte GST, GR, SOD and Catalase levels in PT, CR and NR

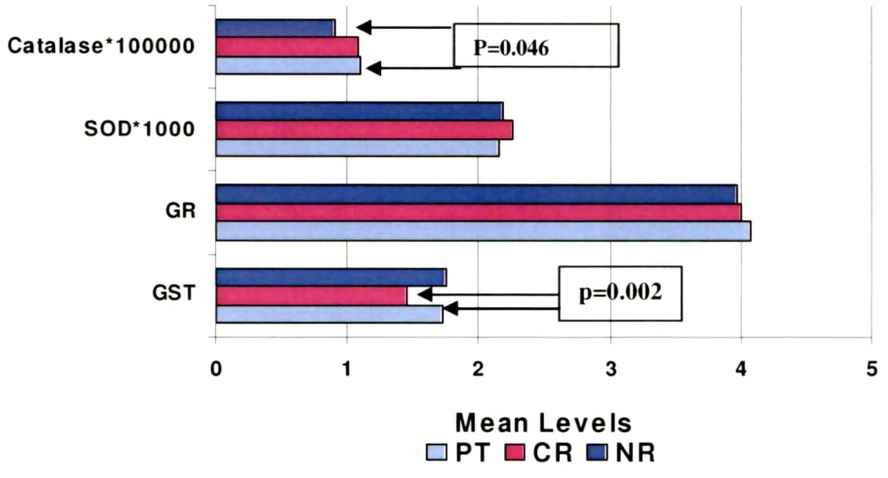


Figure-4.2.53 Comparison of plasma GST, GR and thiol levels between PT, CR and NR

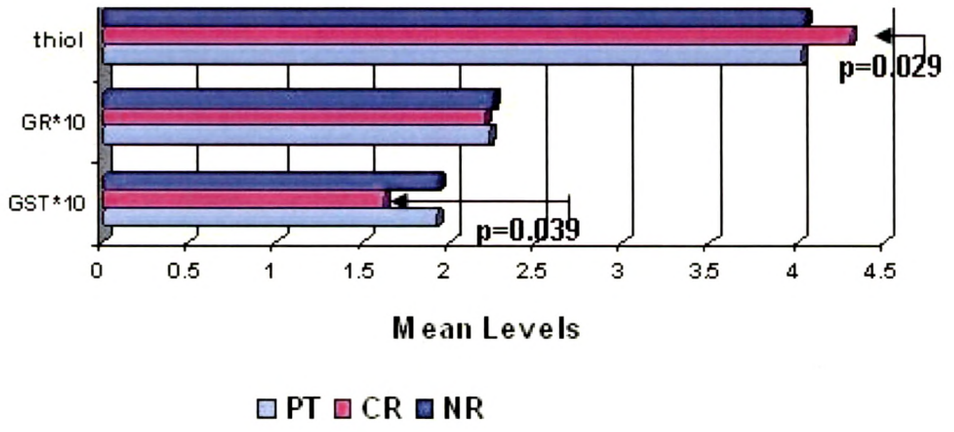


Figure-4.2.53 shows comparison of plasma GST, GR and thiol with treatment response. Plasma thiol, GR and GST levels were comparable between NR and PT. Mean values of plasma thiol were significantly higher in CR than PT (p=0.029). Plasma GR levels were comparable between CR and PT. Plasma GST levels were significantly lower in CR as compared to PT (p=0.039).