Results

4.1 MULTIPLE MYELOMA PATIENTS

4.1.1 Serum total protein electrophoresis

Serum protein electrophoresis using agar gel revealed protein separation into albumin, alpha-1, alpha-2, beta and gamma globulin fractions. These protein fractions and their relative quantity were the primary focus of the interpretation of serum protein electrophoresis. The electrograms were scanned and band intensity of the protein fractions were obtained by densitometric analysis.

Figure-1: Representative patterns of agarose gel electrophoresis in controls and MM patients

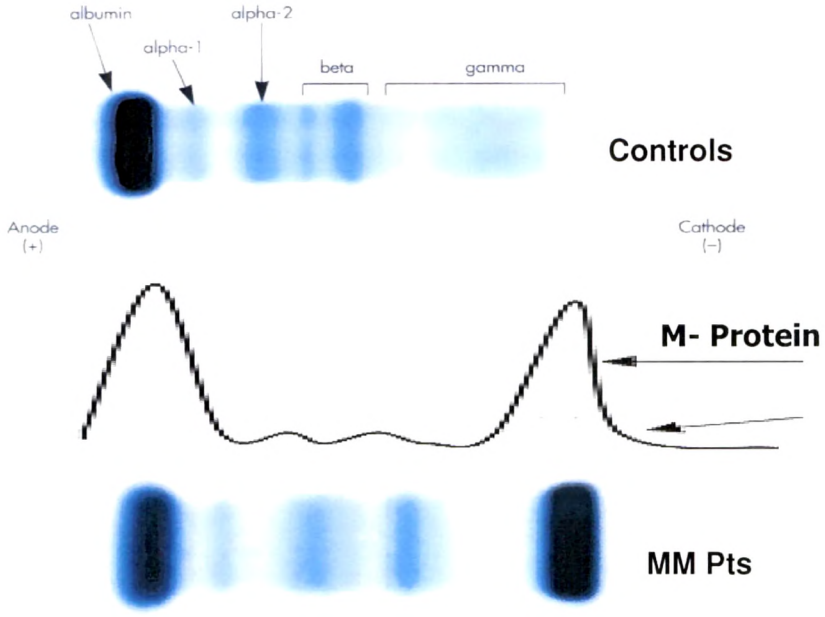


Figure: 1 shows representative electrophoretic patterns in controls and MM patients. 58% of the MM patients showed a large and narrow spike in the gamma or beta-gamma (overlapping) region. The protein band was termed as M-protein (Multiple Myeloma protein). The MM patients were further classified into two groups:

Group-I: MM patients with presence of M-protein (N=29).

Group-II: MM patients with absence of M-protein (N=21).

Figure-2: Comparison of serum protein fractions by agarose gel electrophoresis between controls and MM patients

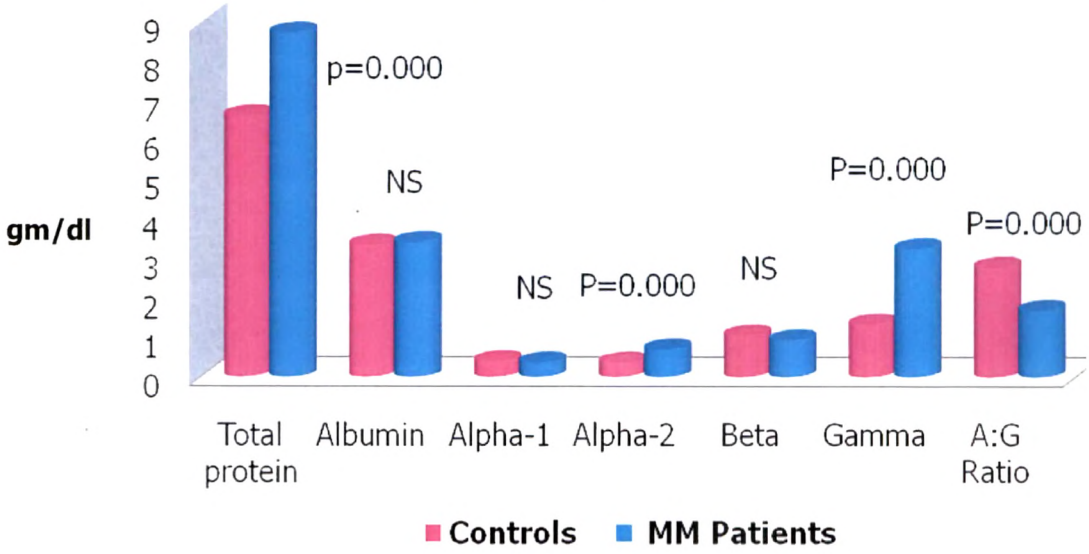
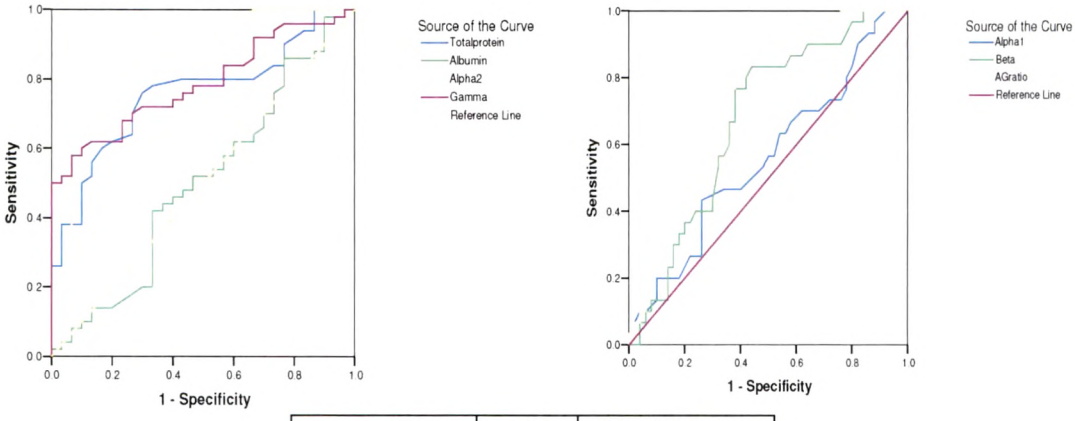


Figure: 2 shows the comparison of protein fractions between controls and MM patients. Mean serum total protein, alpha-2 and gamma fractions were significantly elevated (**p=0.000, p=0.000** and **p=0.000**; respectively) in MM patients as compared to the controls. Serum A:G ratio was significantly decreased (**p=0.000**) in MM patients as compared to the controls. Alterations in serum albumin, alpha-1 and beta levels were comparable between controls and MM patients.

ROC curve analysis: ROC curve analysis is more meaningful way to evaluate discriminatory efficacy of the parameters between two groups to be studied. It provides comparison of sensitivity and specificity of the parameters simultaneously. ROC curves were constructed for all the parameters to evaluate their efficacy to discriminate between controls and MM patients.

ROC curve were plotted for serum protein fractions separated by agarose protein gel electrophoresis. As evident from **figure: 3**, serum total protein, alpha-2, beta, gamma and A:G ratio could significantly (**p=0.000, p=0.000, p=0.009, p=0.000** and **p=0.000**; respectively) discriminate between controls and MM patients. The AUC for serum total protein, alpha-2, beta, gamma and A:G ratio were 0.757, 0.884, 0.674, 0.785 and 0.749, respectively.

Figure-3: ROC curve for serum protein fractions between controls and MM patients



Parameters	AUC	Significance
Total protein	0.757	**p=0.000**
Albumin	0.499	p=0.988
Alpha1	0.560	p=0.374
Alpha-2	0.884	**p=0.000**
Beta	0.674	**p=0.009**
Gamma	0.785	**p=0.000**
A:G ratio	0.749	**p=0.000**

*AUC: Area under curve

Figure-4: Comparison of serum protein fractions between group-I and group-II MM patients

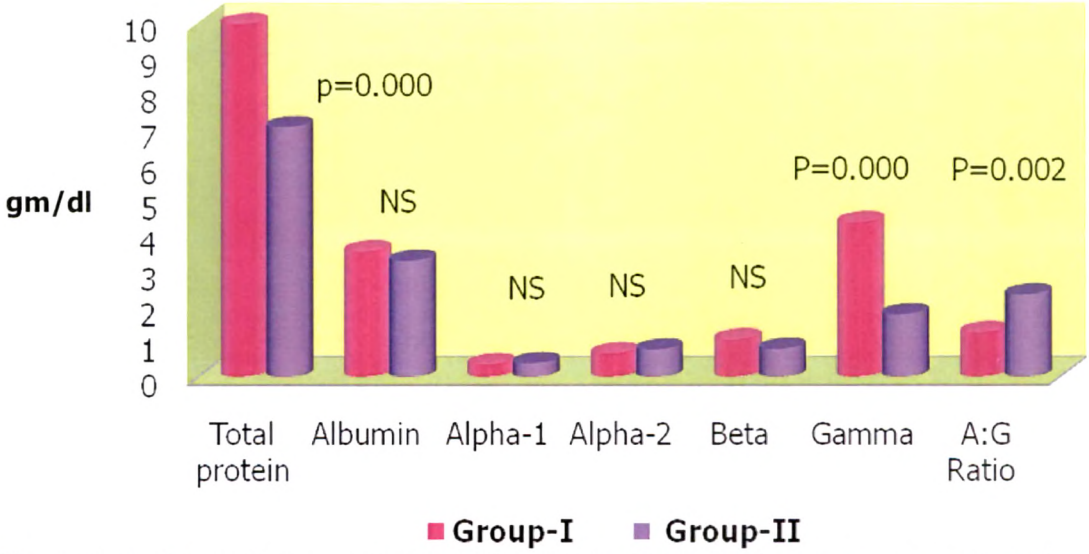
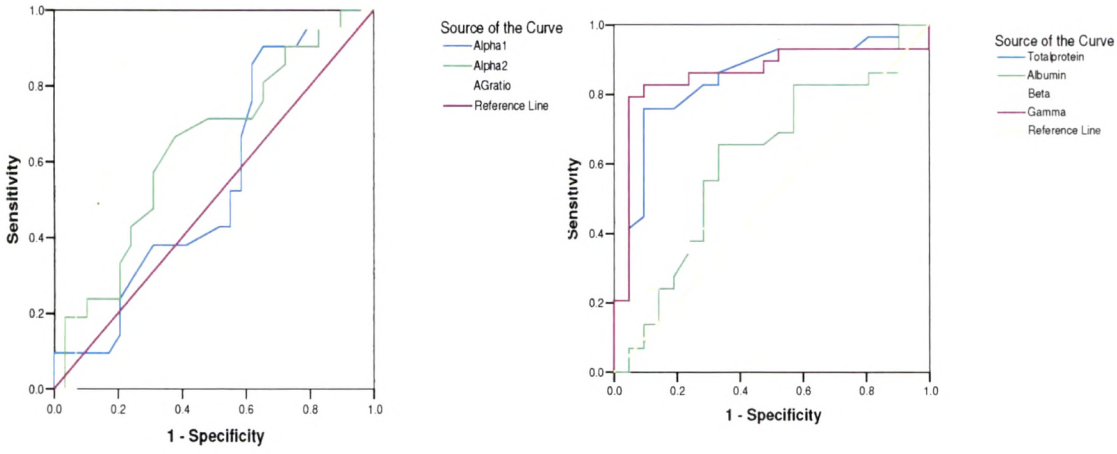


Figure: 4 shows the comparison of protein fractions between group-I and group-II MM patients. Mean serum total protein and gamma fractions were significantly elevated (**p=0.000** and **p=0.000**; respectively) in group-I MM patients as compared to group-II patients. Serum A:G ratio were significantly

higher (**p=0.002**) in group-II MM patients as compared to group-I MM patients. Serum albumin, alpha-1, alpha-2 and beta levels were comparable between the two groups of MM patients.

Figure-5: ROC curve for serum protein fractions between group-I and group-II MM patients



Parameters	AUC	Significance
Total protein	0.842	**p=0.000**
Albumin	0.387	P=0.175
Alpha-1	0.451	P=0.555
Alpha-2	0.640	P=0.095
Beta	0.556	p=0.504
Gamma	0.857	**p=0.000**
A:G ratio	0.821	**P=0.000**

*AUC: Area under curve

As evident from **figure: 5**, serum total protein, gamma fraction and A:G ratio could significantly discriminate (**p=0.000, AUC=0.842; p=0.000, AUC=0.857** and **p=0.000, AUC=0.821;** respectively) between group-I and group-II MM patients.

4.1.2 Serum immunoprofiling in controls and MM patients

Serum immunoprofiling was performed in controls and MM patients in order to evaluate immunoglobulin status in MM patients.

Figure-6: Representative diagram of immunoprofiling in controls and MM patients

Controls **MM Patients**

Figure: 6 shows the immunoglobulin levels analysed on radial immunodiffusion plates. The immunoglobulins were observed as transparent ring and the ring diameters were measured.

Figure-7: Comparison of serum immunoglobulin levels between controls and MM patients

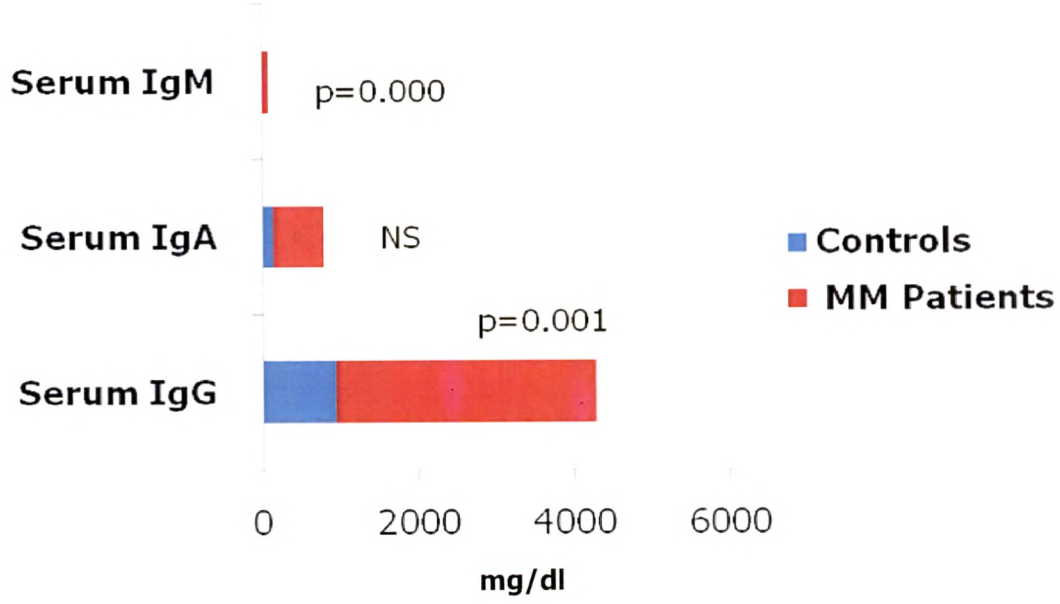


Figure: 7 shows the comparison of the immunoglobulin levels between controls and MM Patients. Mean serum IgG and IgM values were significantly elevated (**p=0.001** and **p=0.000**; respectively) in MM Patients as compared to the controls. Serum IgA level was comparable between controls and MM Patients.

Table-1: Immunoglobulin levels in MM patients

Class	Frequency (%) (29/50)
Monoclonal Band	29 (58%)
IgG	23 (79.4%)
IgA	04 (13.8%)
IgM	02 (6.8%)
M-Protein concentration	
< 3 gm/dl	09 (31%)
≥3-< 6 gm/dl	13 (45%)
≥6 gm/dl	07 (24%)

As documented in **table-1,** the M-protein was found in 58% of MM patients and also showed that in majority of the patients, M-protein were of IgG type (79.4%). The protein content of M-protein in MM patients were ≥3-<6 gm/dl in 45% and ≥6 gm/dl in 24% of MM patients.

4.1.3 Serum protein profiling by native-PAGE:

Protein profiling by native-PAGE is a specialized form of identification of proteins in normal and diseased serum samples.

Figure: 8 shows representative electrophoretic patterns of controls and MM patients. The serum native-PAGE for protein profiling revealed 6 major fractions viz. UnLMW, prealbumin, albumin, alpha, beta and gamma fractions which were scanned and each protein fractions value were calculated.

Figure: 9 shows the comparison of serum native-PAGE profiles between controls and MM patients. Mean serum total protein, UnLMW, prealbumin, albumin, alpha, and gamma values were significantly higher ($p=0.000$, $p=0.016$, $p=0.008$ $p=0.012$, $p=0.002$ and $p=0.000$; respectively) in MM patients as compared to the controls.

Figure-8: Representative patterns of serum protein profiling by native-PAGE in controls and MM patients

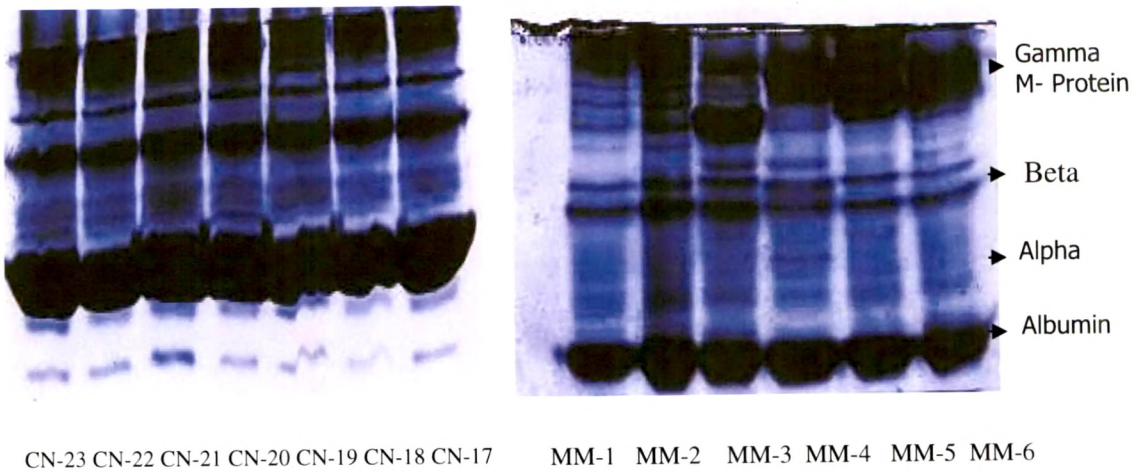
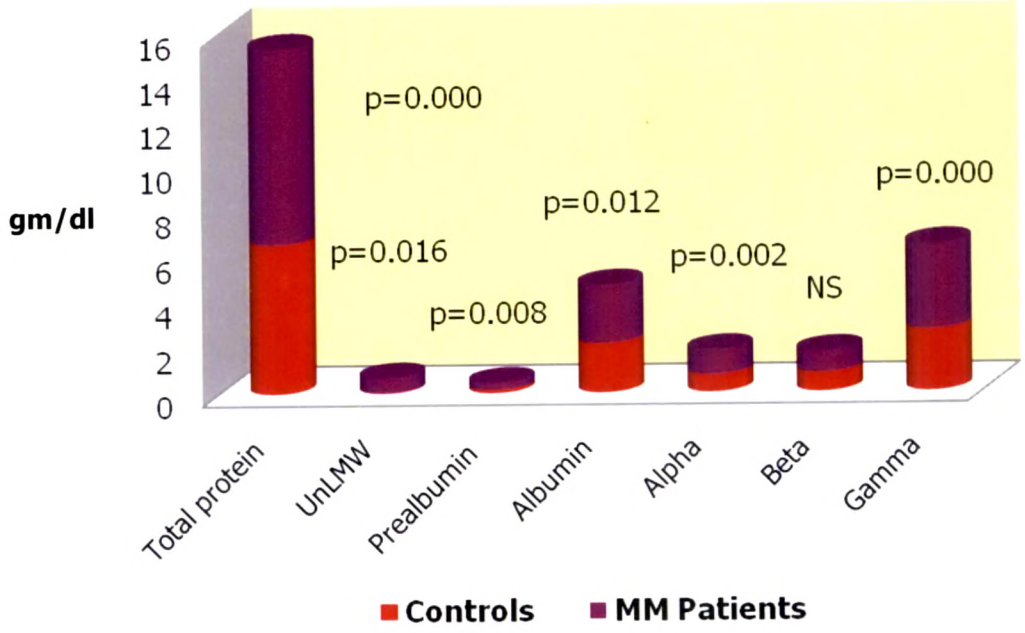
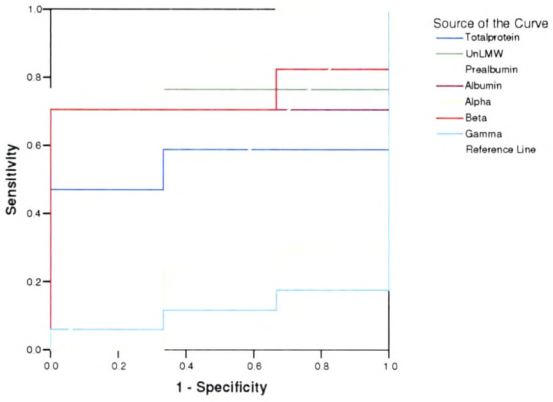


Figure-9: Comparison of serum native-PAGE profiles between controls and MM patients



ROC Analysis: ROC curve were plotted for serum native-PAGE profiles to evaluate their efficacy to discriminate between controls and MM patients.

Figure-10: ROC curve for serum protein profiles in controls and MM patients



Parameters	AUC	Significance
Total protein	0.549	p=0.791
UnLMW	0.765	p=0.153
Prealbumin	0.882	**p=0.039**
Albumin	0.706	p=0.266
Alpha	0.176	p=0.081
Beta	0.745	p=0.186
Gamma	0.118	p=0.039

*AUC: Area under curve

As evident from **figure: 10**, serum prealbumin (**p=0.039, AUC=0.882**) could significantly discriminate between controls and MM patients.

Figure-11: Comparison of serum native-PAGE profiles between group-I and group-II MM patients

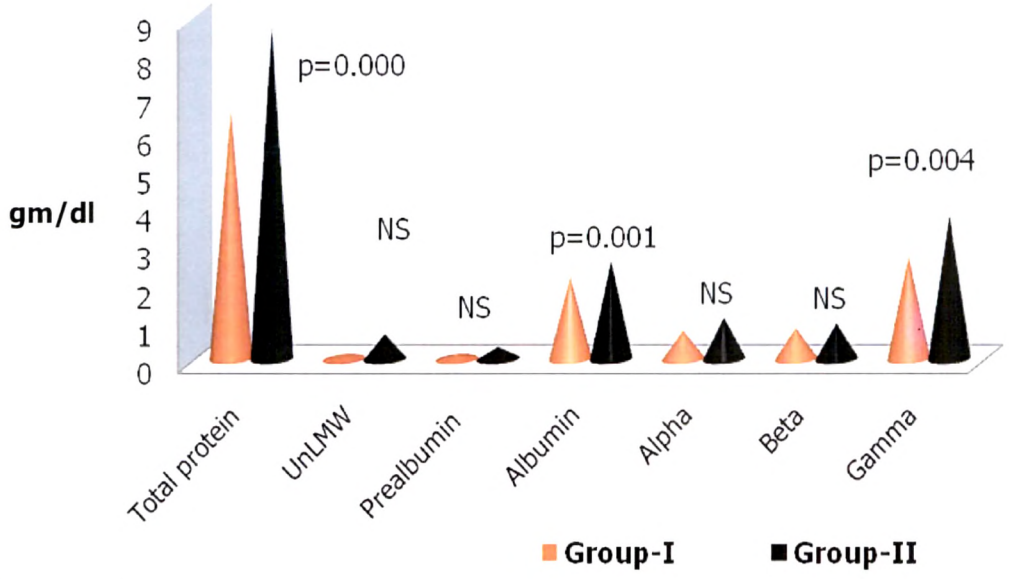
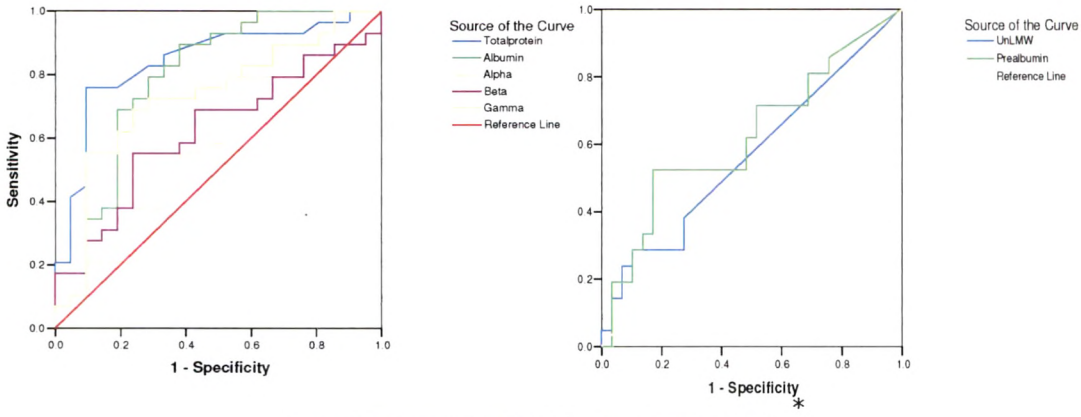


Figure: 11 shows the comparison of serum total protein profiles between group-I and group-II MM patients. Mean serum total protein, albumin and gamma values were significantly higher (**p=0.000, p=0.001** and **p=0.004**; respectively) in group-II as compared to the group-I MM patients.

ROC analysis: As documented in **figure: 12**, serum total protein and gamma (**p=0.000, AUC=0.842** and **p=0.006, AUC=0.731**; respectively) could significantly discriminate between group-I and group-II MM patients.

Figure-12: ROC curve for serum protein profiles in group-I and group-II MM patients



Parameters	AUC	Significance
Total protein	0.842	**p=0.000**
UnLMW	0.564	p=0.443
Prealbumin	0.622	p=0.146
Albumin	0.588	p=0.293
Alpha	0.688	p=0.143
Beta	0.622	p=0.059
Gamma	0.731	**p=0.006**

*AUC: Area under curve

Figure-13: Comparison of serum native-PAGE profiles ratio between controls and MM patients

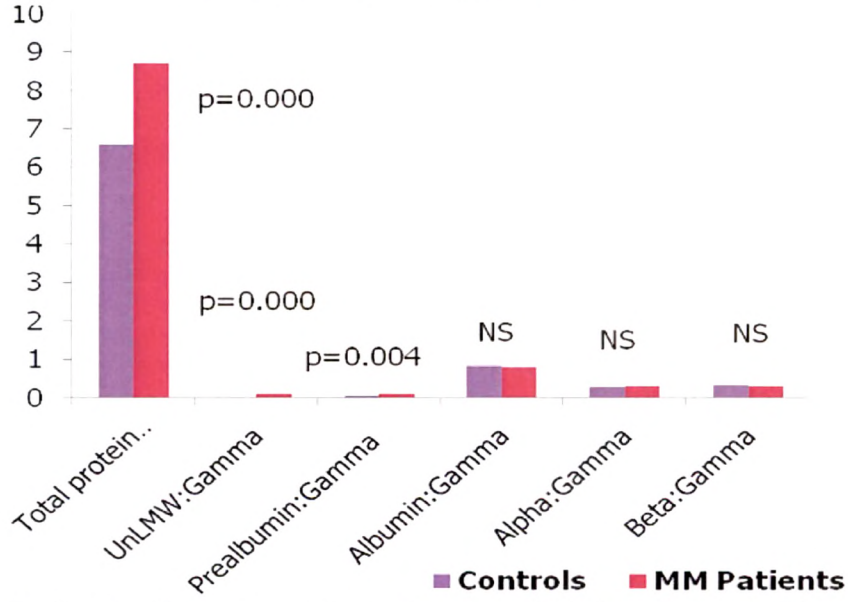
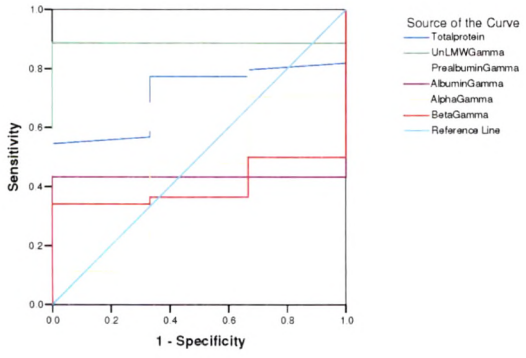


Figure: 13 depicts the comparison of serum total protein profiles ratio between controls and MM patients. Mean serum total protein, UnLMW:gamma and pre albumin:gamma values were significantly higher (**p=0.000, p=0.000** and **p=0.004**; respectively) in MM patients as compared to the

controls. Mean values of serum albumin:gamma, alpha:gamma and beta:gamma were comparable between controls and MM patients.

Figure: 14 Comparison of serum protein profiles ratio between controls and MM patients



Parameters	AUC	Significance
Total protein	0.712	p=0.223
UnLMW:gamma	0.886	**p=0.026**
Prealbumin:gamma	0.712	p=0.223
Albumin:gamma	0.432	p=0.695
Alpha:gamma	0.432	p=0.695
Beta:gamma	0.402	p=0.572

*AUC: Area under curve

As evident from **figure: 14,** ratio of UnLMW:gamma (**p=0.026, AUC=0.886**) could significantly discriminate between controls and MM patients.

Figure-15: Comparison of serum native-PAGE profiles ratio between group-I and group-II MM patients

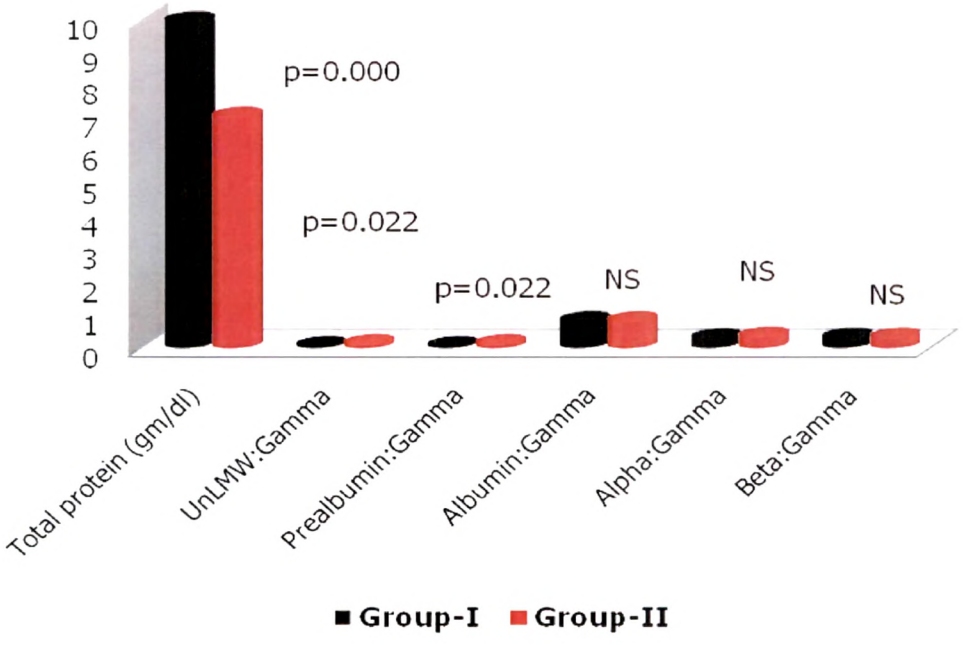
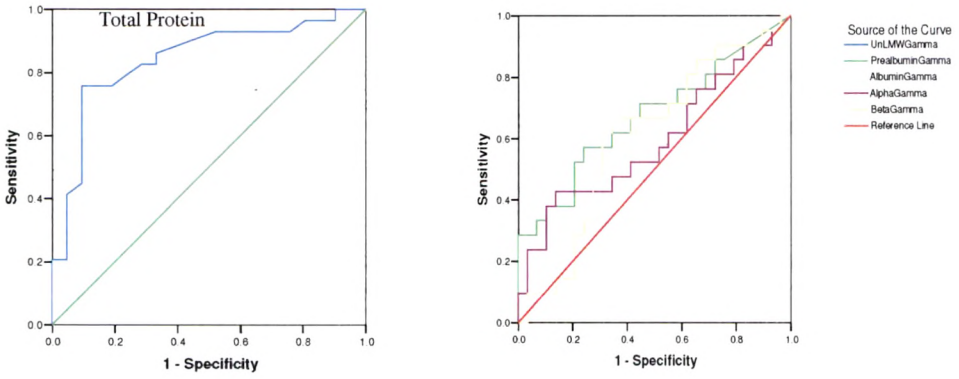


Figure: 15 depicts the comparison of serum total protein profiles ratio between group-I and group-II MM patients. Mean serum total protein, UnLMW:gamma and prealbumin:gamma values were significantly elevated

(**p=0.000, p=0.022** and **p=0.022**; respectively) in group-I as compared to the group-II MM patients.

Figure-16: ROC curve for comparison between group-I and group-II MM patients



Parameters	AUC	Significance
Total protein	0.842	**p=0.000**
UnLMW:gamma	0.672	**p=0.039**
Prealbumin:gamma	0.672	**p=0.039**
Albumin:gamma	0.499	P=0.992
Alpha:gamma	0.594	P=0.258
Beta:gamma	0.609	P=0.191

*AUC: Area under curve

As evident from **figure: 16**, serum total protein, UnLMW:gamma and prealbumin:gamma could significantly (**p=0.000, AUC 0.842**; **p=0.039, AUC 0.672** and **p=0.039, AUC 0.672**; respectively) discriminate between group-I and group-II MM patients.

4.1.4 Glycosylation Changes in MM Patients

Glycosylation, i.e. the attachment of monosaccharides or extended sugar chains to proteins, represents the most well-defined and most complex form of post-translational modifications.

Figure-17: Comparison of serum levels of glycoconjugates and protein ratio of glycoconjugates between controls and MM patients

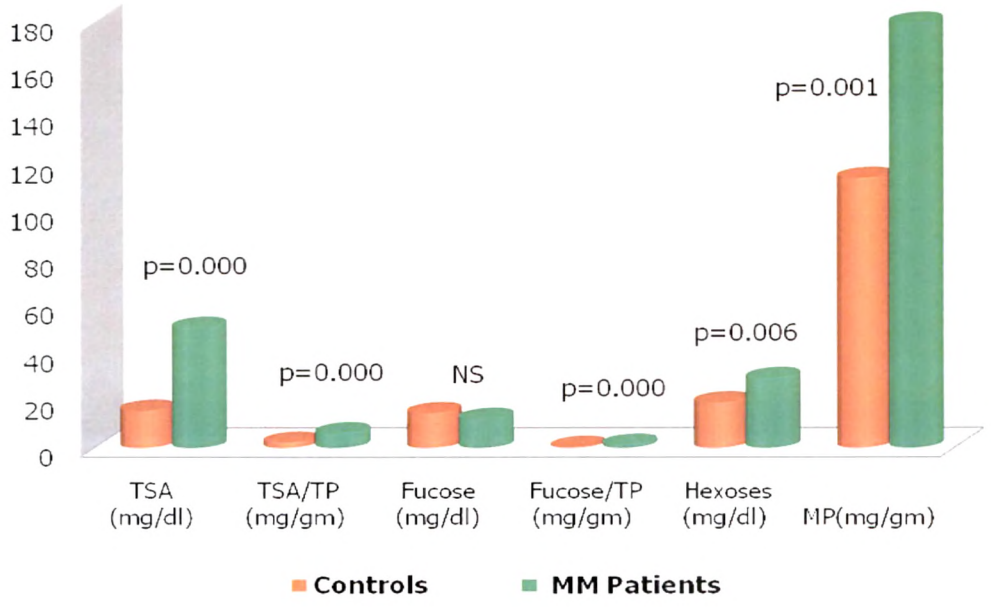
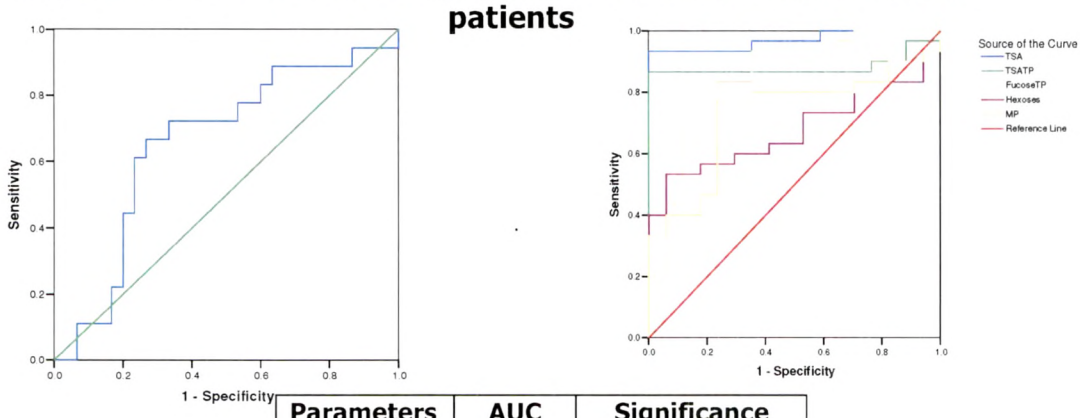


Figure: 17 shows the comparison of the glycoconjugates and protein ratio of glycoconjugates between controls and MM patients. Serum TSA, TSA/TP, fucose/TP, hexoses and MP levels were significantly higher (**p=0.000, p=0.000, p=0.000, p=0.006** and **p=0.001;** respectively) in MM patients as compared to the controls.

Figure-18: ROC curve for comparison between controls and MM patients



Parameters	AUC	Significance
TSA	0.969	**p=0.000**
TSA/TP	0.882	**p=0.000**
Fucose	0.656	p=0.074
Fucose/TP	0.851	**p=0.000**
Hexoses	0.678	**p=0.044**
MP	0.725	**p=0.011**

*AUC: Area under curve

ROC Analysis: As evident from **figure: 18**, serum TSA, TSA/TP, fucose/TP, hexoses and MP could significantly (**p=0.000, AUC=0.969**; **p=0.000, AUC=0.882**; **p=0. 000, AUC=0.851**; **p=0.044, AUC=0.678** and **p=0.011, AUC=0.725**; respectively) discriminate between controls and MM patients.

Pearson's Correlation Coefficient: Pearson's correlation coefficient is a measure of linear association between two variables. Values of the correlation coefficient range from -1 to 1. The sign of the coefficient indicates the direction of the relationship and its absolute value indicates the strength, with larger absolute values indicating stronger relationships.

Correlation of glycoconjugates and protein ratio of glycoconjugates between controls and MM patients

Table-2 shows correlation coefficients of glycoconjugates and protein ratio of glycoconjugates between controls and MM patients. Serum TSA, fucose and hexoses showed significant positive correlation (**p=0.000, p=0.004** and **p=0.000**; respectively) with TSA/TP, fucose/TP and MP, respectively.

Table-2: Correlation between serum glycoconjugates and protein ratio of glycoconjugates

Parameters	TSA/TP
TSA	**r = 0.887; p=0.000**
	Fucose/TP
Fucose	**r = 0.404; p=0.004**
	MP
Hexoses	**r = 0.613; p=0.000**

Figure-19: Comparison of serum levels of glycoconjugates and protein ratio of glycoconjugates between group-I and group-II MM patients

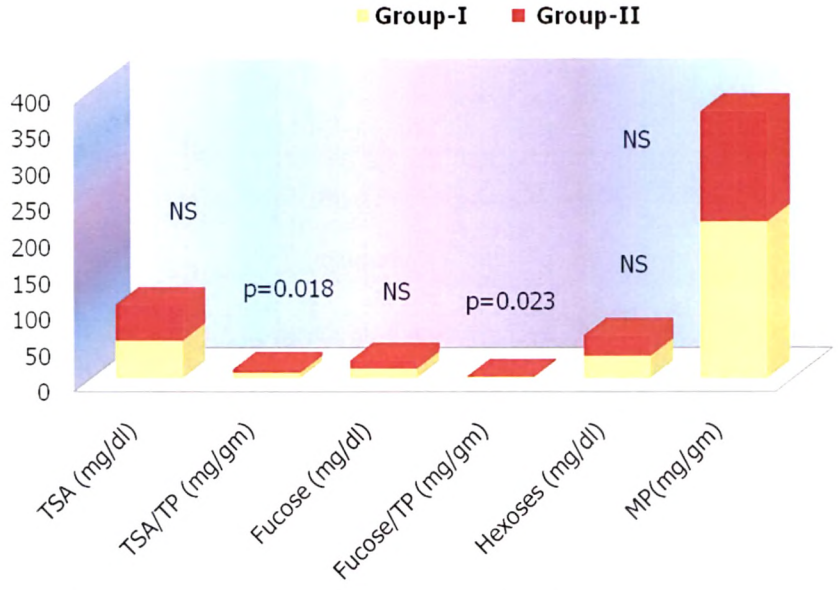
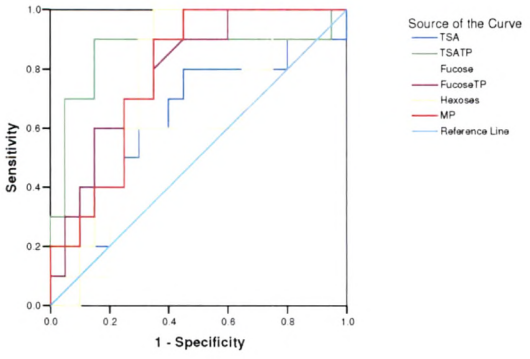


Figure: 19 shows comparison of serum levels of glycoconjugates and protein ratio of glycoconjugates between group-I and group-II MM patients. Serum TSA/TP and fucose/TP levels were significantly higher (**p=0.018** and **p=0.023**; respectively) in group-II as compared to the group-I MM patients.

ROC Analysis: Serum TSA/TP, fucose/TP, hexoses and MP levels could significantly (**p=0.002, AUC=0.855**; **p=0.011, AUC=0.790**; **p=0.016, AUC=0.775** and **p=0.012, AUC=0.785**; respectively) discriminate between group-I and group-II MM patients (**figure: 20**).

Figure-20: ROC curve for comparison between group-I and group-II MM patients



Parameters	AUC	Significance
TSA	0.620	p=0.291
TSA/TP	0.855	**p=0.002**
Fucose	0.600	p=0.379
Fucose/TP	0.790	**p=0.011**
Hexoses	0.775	**p=0.016**
MP	0.785	**p=0.012**

*AUC: Area under curve

Correlation of serum glycoconjugates and protein ratio of glycoconjugates between group-I and group-II MM patients

Pearson's correlation coefficients were assessed for serum levels of glycoconjugates and protein ratio of glycoconjugates in group-I and group-II MM patients (**table-3**). Serum TSA, fucose and hexoses showed significant positive correlation (**p=0.000, p=0.000** and **p=0.000**; respectively) with TSA/TP, fucose/TP and MP.

Table-3: Correlation between serum glycoconjugates and protein ratio of glycoconjugates

Parameters	TSA/TP
TSA	**r =0.832; p=0.000**
	Fucose/TP
Fucose	**r = 0.892; p=0.000**
	MP
Hexoses	**r = 0.659; p=0.000**

4.1.5 Glycoprotein profiling in MM patients

Serum glycoprotein profiling by native-PAGE: Native-PAGE electrophoretic separation of serum glycoprotein can provide a comprehensive view on various glycoproteins. Serum glycoprotein profiles in various fractions i.e. prealbumin, albumin, alpha, beta and gamma regions were analyzed from serum.

Figure-21: Representative glycoproteins native-PAGE electrophoretic patterns in controls and MM patients

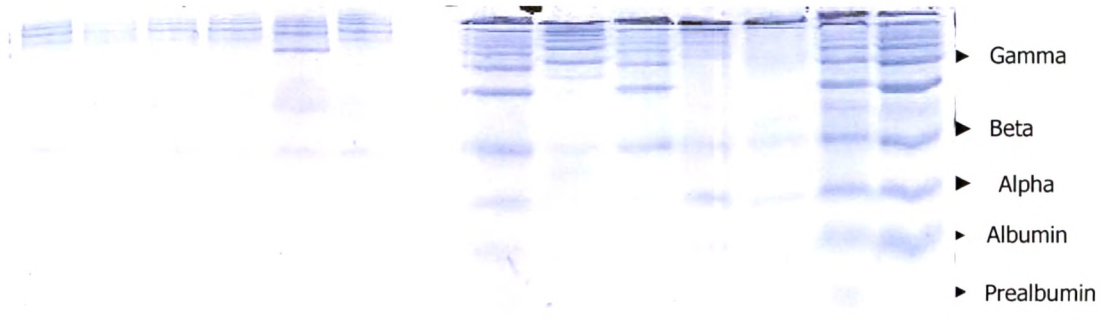
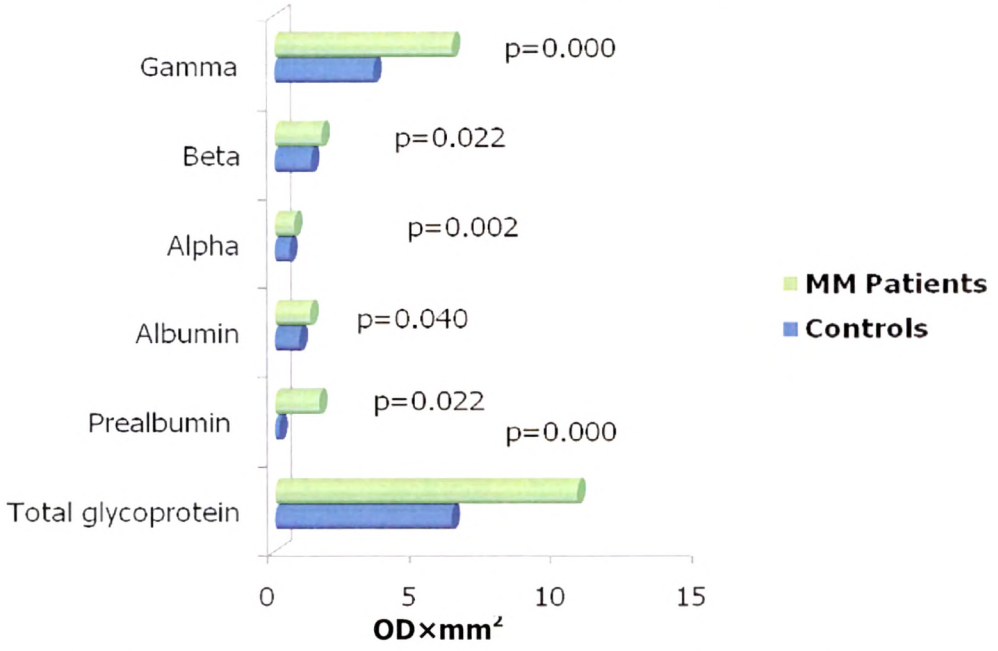


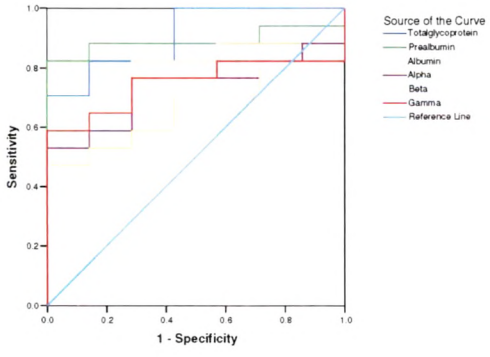
Figure: 21 shows representative glycoprotein electrophoretic patterns in controls and MM patients. The serum native-PAGE for glycoprotein profiling revealed 5 major fractions viz. prealbumin, albumin, alpha, beta and gamma fractions which were scanned and each protein fractions value were calculated.

Figure-22: Comparison of serum glycoprotein profiles (native-PAGE) between controls and MM patients



As depicted in **figure: 22**, serum total glycoprotein, prealbumin, albumin, alpha, beta and gamma glycoproteins were found to be significantly elevated (**p=0.000, p=0.022, p=0.040, p=0.002, p=0.022** and **p=0.000**; respectively) in MM patients as compared to the controls.

Figure-23: ROC curve for comparison of glycoprotein profiles between controls and MM patients

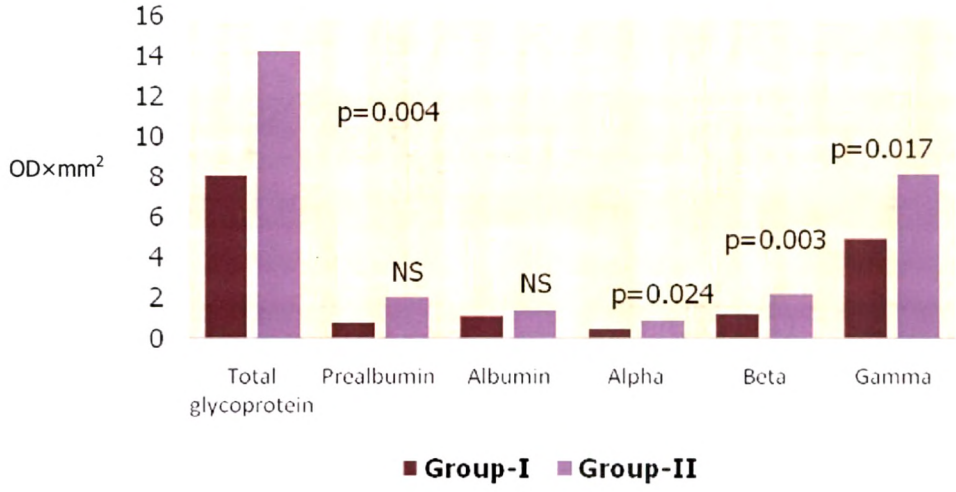


Parameters	AUC	Significance
Total glycoprotein	0.908	**p=0.002**
Prealbumin	0.891	**p=0.003**
Albumin	0.756	**p=0.053**
Alpha	0.731	p=0.081
Beta	0.664	p=0.216
Gamma	0.748	p=0.061

*AUC: Area under curve

As evident from **figure: 23**, serum total glycoprotein, prealbumin and albumin could significantly (**p=0.002, AUC=0.908; p=0.003, AUC=0.891** and **p=0.053, AUC=0.756**; respectively) discriminate between controls and MM patients.

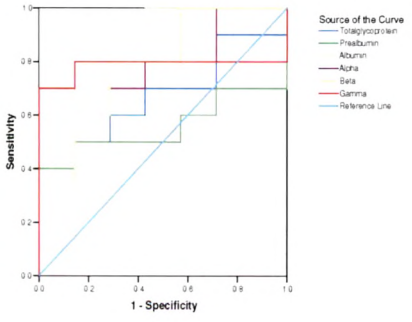
Figure-24: Comparison of serum glycoprotein profiles (native-PAGE) between group-I and group-II MM patients



Serum total glycoprotein, alpha, beta and gamma glycoproteins were found to be significantly elevated (**p=0.004, p=0.024, p=0.003** and **p=0.017**; respectively) in group-II as compared to the group-I MM patients (**figure: 24**).

ROC Analysis: As documented in **figure: 25,** serum albumin, beta and gamma could significantly (**p=0.040, AUC=0.800**; **p=0.051, AUC=0.786** and **p=0.051, AUC=0.786**; respectively) discriminate between glycoprotein profiles in group-I and group-II MM patients.

Figure-25: ROC curve for comparison of glycoprotein profiles between group-I and group-II MM patients



Parameters	AUC	Significance
Total glycoprotein	0.671	p=0.242
Prealbumin	0.557	p=0.696
Albumin	0.800	**p=0.040**
Alpha	0.743	p=0.097
Beta	**0.786**	**p=0.051**
Gamma	**0.786**	**p=0.051**

*AUC: Area under curve

Figure-26: Comparison of ratio of serum glycoprotein fractions between controls and MM patients

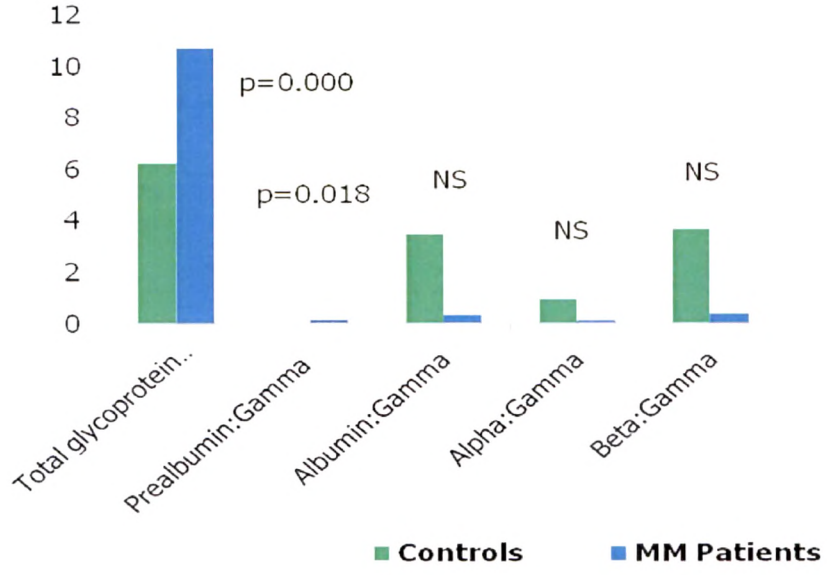
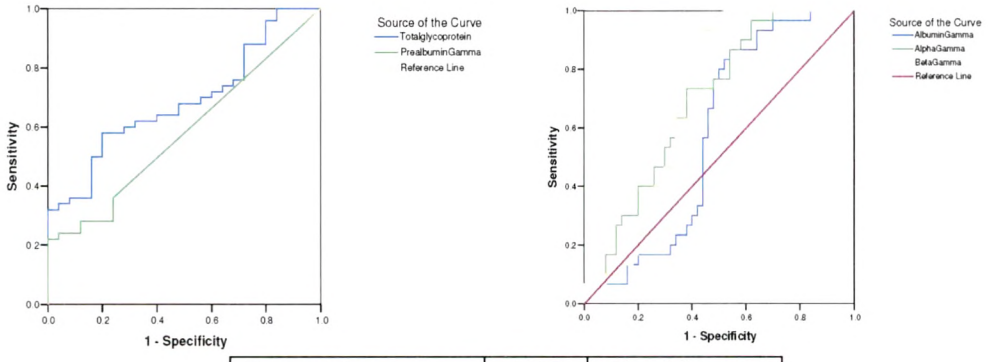


Figure: 26 shows the comparison of serum glycoprotein ratio of fractions in controls and MM patients. Serum total glycoprotein and prealbumin:gamma fractions were found to be significantly higher (**p=0.000** and **p=0.018**; respectively) in MM patients as compared to the controls.

Figure-27: ROC curve for comparison between controls and MM patients

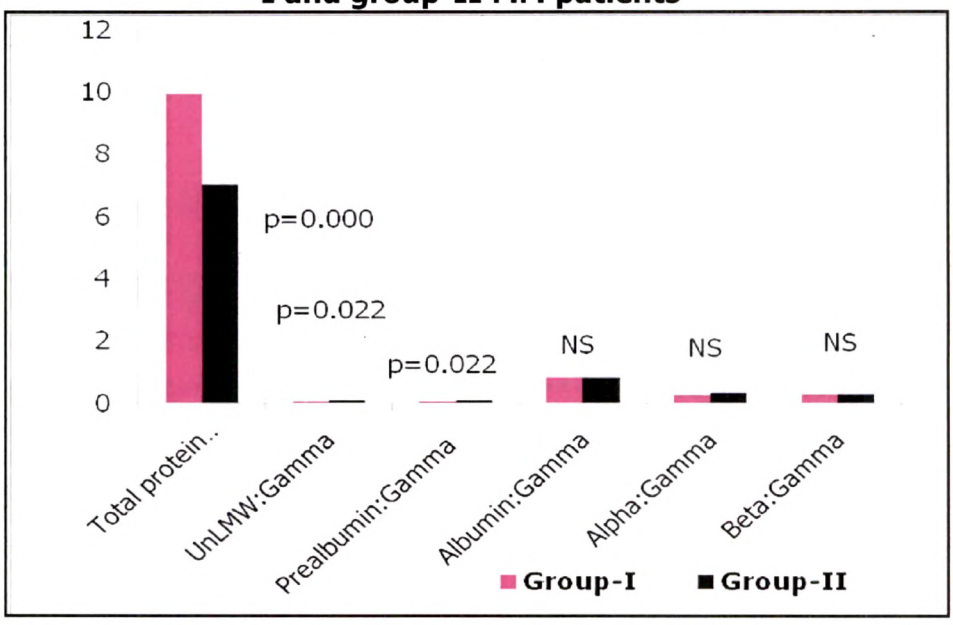


Parameters	AUC	Significance
Total glycoprotein	0.686	**p=0.009**
Prealbumin:gamma	0.578	p=0.271
Albumin:gamma	0.578	p=0.245
Alpha:gamma	0.693	**p=0.004**
Beta:gamma	0.717	**p=0.001**

*AUC: Area under curve

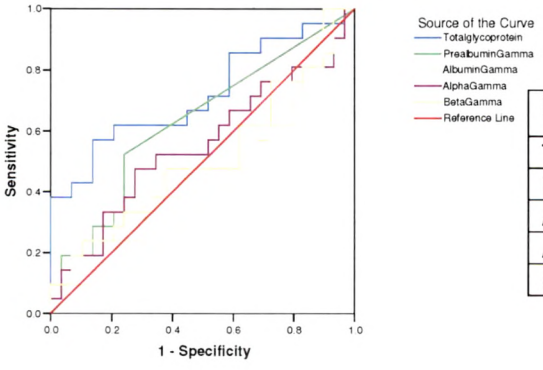
As evident from **figure: 27**, serum total glycoprotein, alpha:gamma and beta:gamma could significantly (**p=0.009, AUC=0.686**; **p=0.004, AUC =0.693** and **p=0.001, AUC=0.717**; respectively) discriminate between controls and MM patients.

Figure-28: Comparison of serum glycoprotein ratio between group-I and group-II MM patients



Serum total glycoprotein levels, UnLMW:gamma and prealbumin:gamma were found to be significantly higher (**p=0.000, p=0.022** and **p=0.022**; respectively) in group-II as compared to group-I MM patients (**figure: 28**).

Figure-29: ROC curve for comparison of glycoprotein profiles between group-I and group-II MM patients



Parameters	AUC	Significance
Total glycoprotein	0.719	**p=0.009**
Prealbumin:gamma	0.632	p=0.114
Albumin:gamma	0.468	p=0.702
Alpha:gamma	0.553	p=0.523
Beta:gamma	0.507	p=0.930

*AUC: Area under curve

ROC Analysis: As evident from **figure: 29,** serum total glycoprotein could significantly (**p=0.009, AUC=0.719**) discriminate between group-I and group-II MM patients.

4.1.6 Serum MMP-2 and MMP-9 levels in controls and MM patients

MMP-2 and MMP-9 were analysed using gelatin zymography (substrate zymography). This method identifies MMPs by the degradation of the substrate and by comparision for their molecular weight. For the analysis, serum aliquots were prepared and protein contents were estimated. 50µg of protein samples were loaded on each lane.

Figure-30: Representative zymogramms of MMP-2(a) and MMP-9(b) latent and active standards

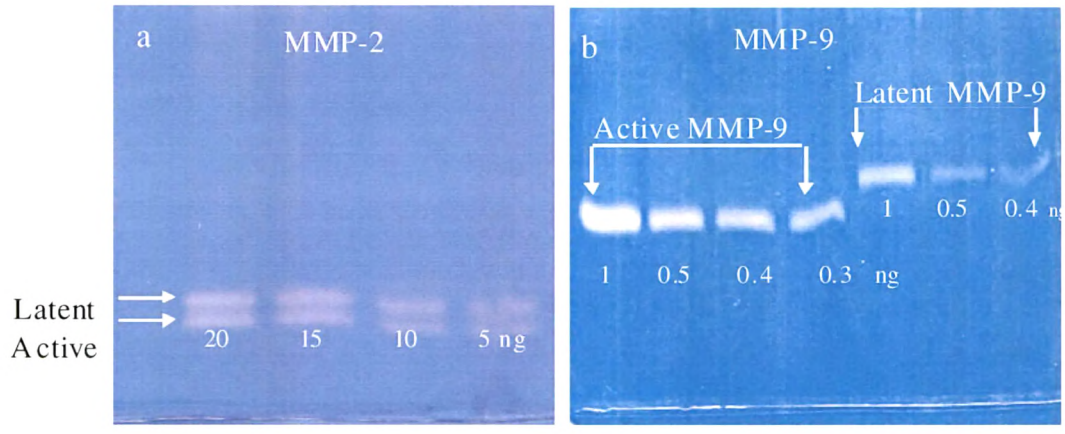
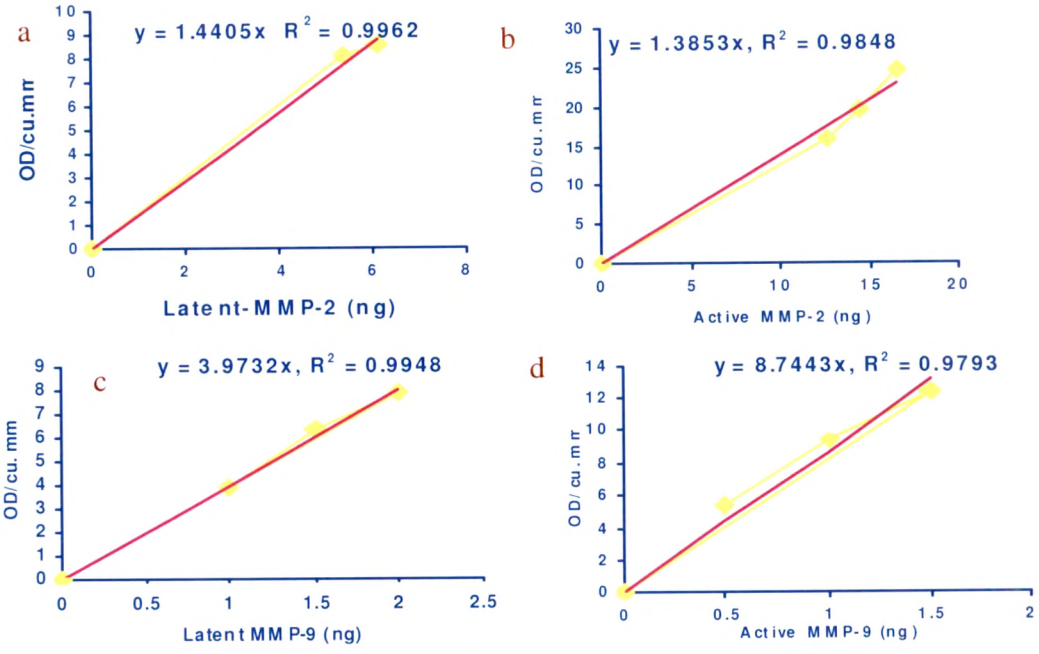


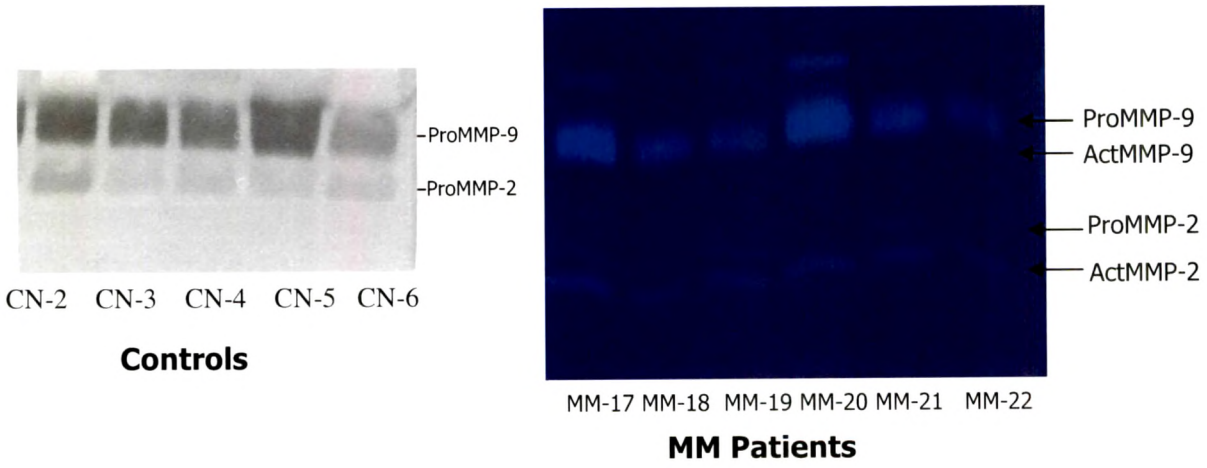
Figure: 30 shows latent (pro) and active MMP-2 and MMP-9 standards (calbiochem, California, USA) which were separated by substrate (gelatin) zymography. The standards were run for gelatin zymography from 0-20 ng and 0-2 ng for latent and active forms of MMP-2 and MMP-9, respectively. Pro and active forms of MMP-2 and MMP-9 were observed as transparent band against undigested blue background.

Figure-31: Standard graphs for MMP-2 (a; latent, b; active) and MMP-9 (c; latent, d; active)



As shown in **figure: 31,** the densitometric analysis of latent and active forms of MMP-2 and MMP-9 revealed a linear correlation.

Figure-32: Representative zymograms of MMP-2 and MMP-9 in controls and MM patients



The zymograms were scanned and pro and active forms of MMP-2 and MMP-9 levels were quantitated based on the band intensity

Figure-33: Comparison of serum proMMP-2, activeMMP-2, totalMMP-2 and activation ratio of MMP-2 by zymography between controls and MM patients

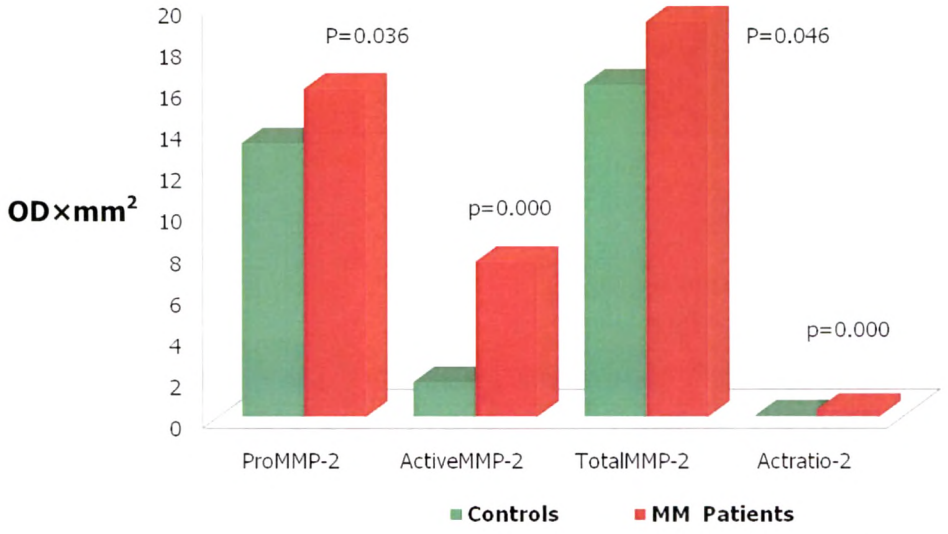


Figure: 33 shows comparison of the levels of pro-, active, total forms and activation ratio of MMP-2 obtained by zymography between controls and MM patients. Serum proMMP-2, activeMMP-2, total MMP-2 and activation ratio of MMP-2 were significantly higher (**p=0.036, p=0.000, p=0.046** and **p=0.000**; respectively) in MM patients as compared to the controls.

Figure-34: Comparison of serum proMMP-9, activeMMP-9, totalMMP-9 and activation ratio of MMP-9 by zymography between controls and MM patients

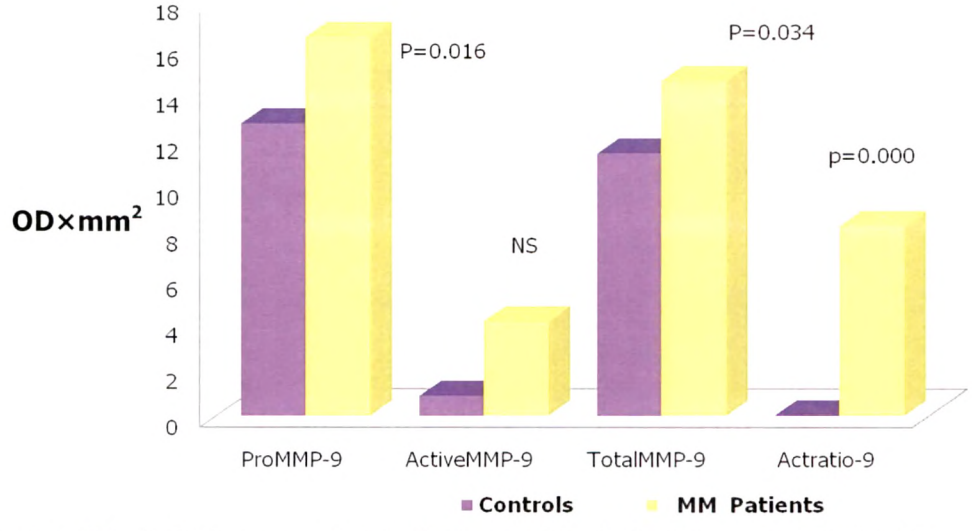
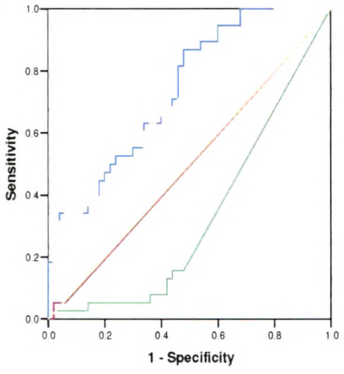


Figure: 34 depicts comparison of pro, active, total forms and activation ratio of MMP-9 obtained by zymography between controls and MM patients. Serum

proMMP-9, totalMMP-9 and activation ratio of MMP-9 were significantly higher (**p=0.016, p=0.034** and **p=0.000**; respectively) in MM patients as compared to the controls. Whereas active MMP-9 were higher in MM patients but mean difference was found not significant.

ROC Analysis:

Figure-35: ROC curve for comparison between controls and MM patients



Parameters	AUC	Significance
ProMMP-2	0.738	**p=0.000**
Active-2	0.554	p=0.411
ProMMP-9	0.550	p=0.448
Active-9	0.456	p=0.503

*AUC: Area under curve

ROC curves were constructed for serum pro- and active forms of MMP-2 and MMP-9 between controls and MM patients. As apparent from **figure: 35**, serum proMMP-2 could significantly (**p=0.000, AUC=0.738**) discriminate between controls and MM patients.

Correlation of serum pro and active forms of MMP-2 and MMP-9 by zymography analysis in MM patients

Correlation coefficient analysis showed that proMMP-2 were found to be negatively and significantly correlated with serum activeMMP-2 and proMMP-9 (**P=0.000** and **P=0.000**; respectively). Whereas proMMP-9 were also negatively and significantly (**P=0.000**) associated with activeMMP-2. No correlation was found between proMMP-9 and activeMMP-9 in MM patients (**table-4**).

Table-4: Correlation between serum pro and active forms of MMP-2 and MMP-9

Parameters	ActiveMMP-2	ProMMP-9
ProMMP-2	**r = -0.545; p=0.000**	**r = -0.533; p=0.000**
	ActiveMMP-9	ActiveMMP-2
ProMMP-9	r = -0.150; p=0.163	**r = -0.381; p=0.000**

Figure-36: Comparison of serum proMMP-2, activeMMP-2, totalMMP-2 and activation ratio of MMP-2 by zymography between group-I and group-II MM patients

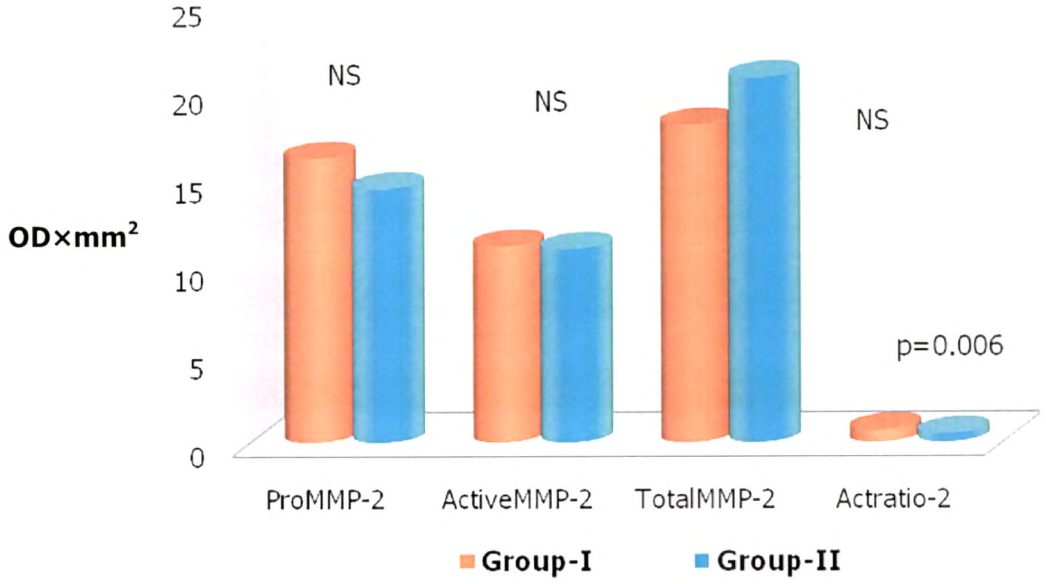


Figure: 36 shows comparison of the levels of pro, active, total forms and activation ratio of MMP-2 obtained by zymography between group-I and group-II MM patients. The activation ratio of MMP-2 was significantly higher (**p=0.006**) in group-I as compared to group-II MM patients.

Figure-37: Comparison of serum proMMP-9, activeMMP-9, totalMMP-9 and activation ratio of MMP-9 by zymography between group-I and group-II MM patients

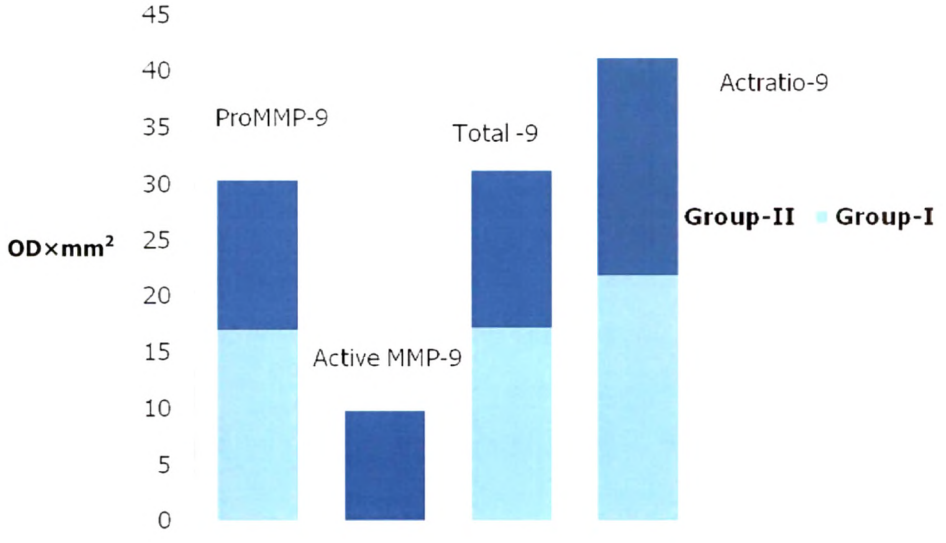


Figure: 37 depicts the comparison of serum pro-, active, total forms and activation ratio of MMP-9 obtained by zymography between group-I and group-II MM patients. Serum pro, active, total forms and activation ratio of MMP-9 were found to be comparable between the two groups of MM patients.

ROC Analysis: ROC curve analysis could not significantly discriminate between serum pro- and active forms of MMP-2 and MMP-9 in group-I and group-II MM patients (**table-5**).

Table-5: Data obtained from ROC curves for the parameters

Parameters	AUC	Significance
ProMMP-2	0.467	p=0.694
Active-2	0.438	p=0.455
ProMMP-9	0.580	p=0.340
Active-9	0.429	p=0.393

*AUC: Area under curve

Correlation of serum pro- and active forms of MMP-2 and MMP-9 by zymography analysis in group-I and group-II MM patients

Correlation coefficient analysis showed that proMMP-2 were found positively and significantly correlated with serum activeMMP-2 and proMMP-9 (**p=0.028** and **p=0.000**; respectively). ProMMP-9 was not correlated with activeMMP-9 and activeMMP-2 in group-I and group-II MM patients (**table-6**).

Table-6: Correlation between serum pro and active forms of MMP-2 and MMP-9

Parameters	ActiveMMP-2	ProMMP-9
ProMMP-2	**r = 0.490; p=0.028**	**r = 0.592; p=0.000**
	ActiveMMP-9	ActiveMMP-2
ProMMP-9	r = -0.163; p=0.399	r = -0.019; p=0.935

4.1.7 Total MMP-2, Total MMP-9, TIMP-1 and TIMP-2

Figure-38: Standard curves for total MMP-2, total MMP-9, TIMP-1, and TIMP-2 by ELISA

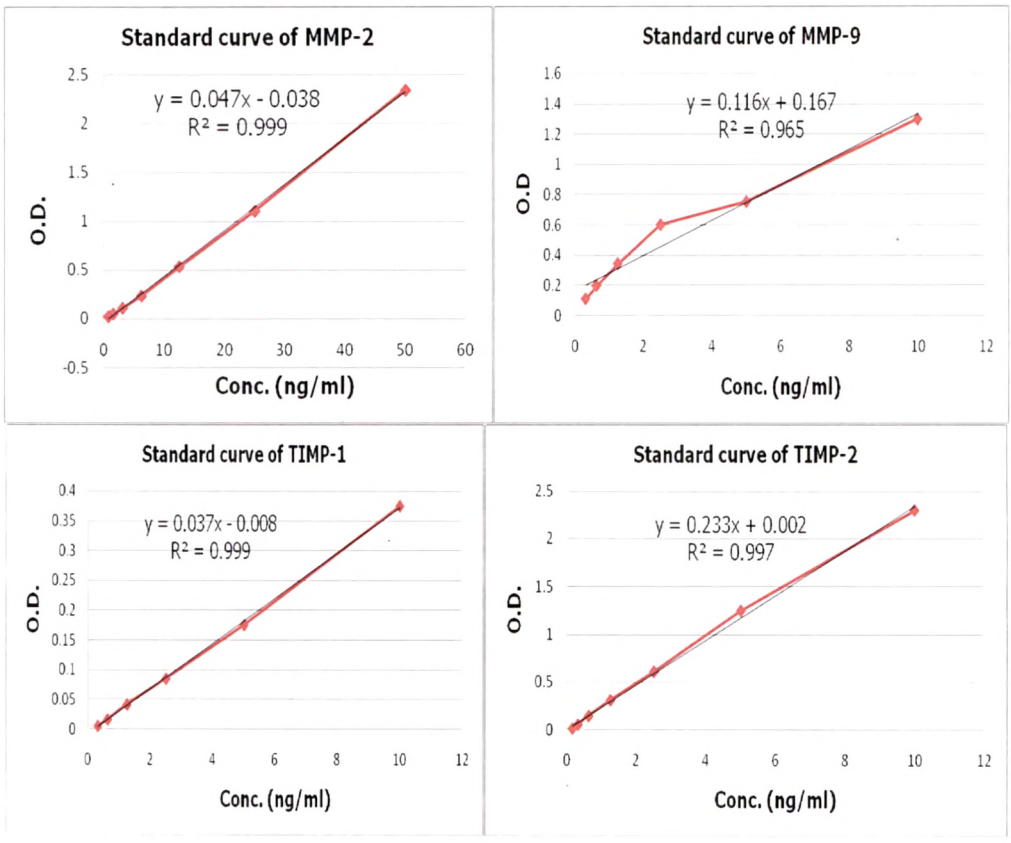


Figure-39: Comparison of serum levels of total MMP-2, total MMP-9, TIMP-1 and TIMP-2 by ELISA between controls and MM patients

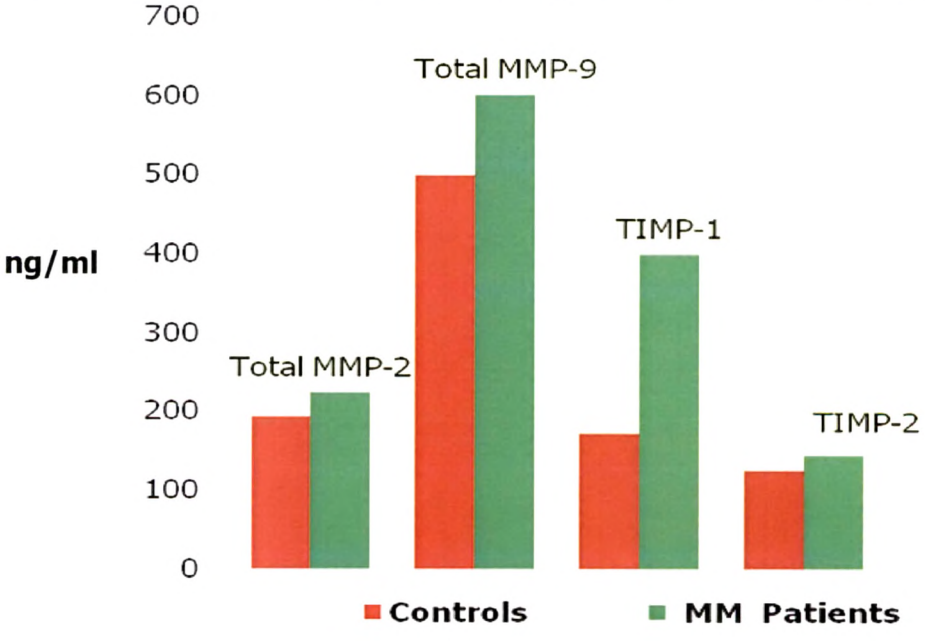
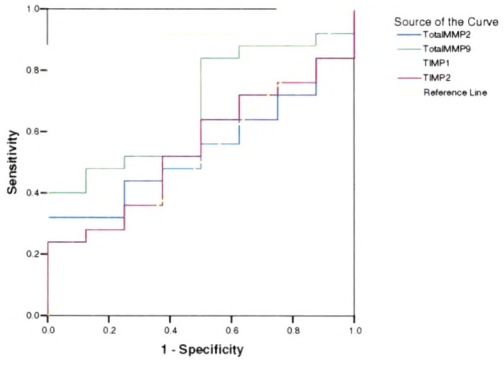


Figure: 38 represents standard curves for MMP-2 and MMP-9 as well as TIMP-1 and TIMP-2. The standard curves showed significant linear correlation.

Figure: 39 shows comparison of serum levels of total MMP-2, total MMP-9, TIMP-1 and TIMP-2 obtained by ELISA between controls and MM patients. Serum TIMP-1 levels were significantly higher (**p=0.000**) in MM patients as compared to the controls. The mean values of serum total MMP-2, total MMP-9 and TIMP-2 were also higher in MM patients as compared to the controls.

ROC Analysis:

Figure-40: Comparison of serum total MMP-2, total MMP-9, TIMP-1 and TIMP-2 in MM patients



Parameters	AUC	Significance
Total MMP-2	0.540	p=0.737
Total MMP-9	0.680	p=0.130
TIMP-1	0.930	**p=0.000**
TIMP-2	0.545	p=0.705

*AUC: Area under curve

As evident from **figure: 40**, serum TIMP-1 could significantly (**p=0.000, AUC=0.930;** respectively) discriminate between controls and MM patients.

Correlation between serum total MMP-2, total MMP-9, TIMP-1 and TIMP-2 in MM Patients

Pearson's correlation was calculated to find out correlation between total MMP-2, total MMP-9, TIMP-1 and TIMP-2. Serum total MMP-2 revealed significantly positive (**p=0.000**) correlation with serum TIMP-2. No correlation was observed between serum total MMP-9 and serum TIMP-1 (**table-7**).

Table-7: Correlation between serum total MMP-2 and TIMP-2 as well as MMP-9 and TIMP-1

Parameters	TIMP-2
Total MMP-2	**r =0.589; p=0.000**
	TIMP-1
Total MMP-9	r =0.024; p=0.887

Figure-41: Comparison of serum levels of total MMP-2, total MMP-9, TIMP-1 and TIMP-2 by ELISA between group-I and group-II MM patients

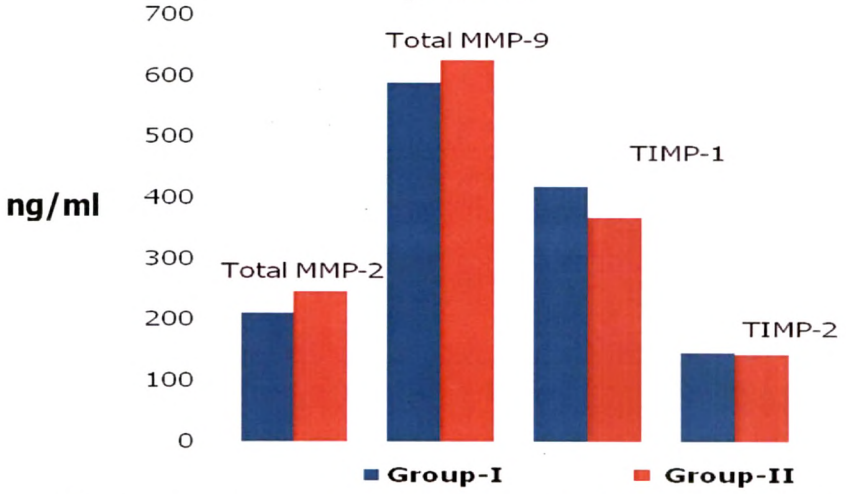


Figure: 41 shows comparison of serum levels of the total MMP-2, total MMP-9, TIMP-1 and TIMP-2 obtained by ELISA between group-I and group-II MM patients. Serum total MMP-2, total MMP-9, TIMP-1 and TIMP-2 mean differences were found to be comparable between group-I and group-II MM patients.

ROC Analysis:

Table-8: Data obtained from ROC curves for the parameters

Parameters	**AUC**	**Significance**
Total MMP-2	0.479	p=0.865
Total MMP-9	0.472	p=0.821
TIMP-1	0.563	p=0.610
TIMP-2	0.500	p=1.000

*AUC: Area under curve

ROC curve analysis could not significantly discriminate between serum total MMP-2, total MMP-9, TIMP-1 and TIMP-2 in group-I and group-II MM patients (**table-8**).

Correlation between serum total MMP-2, total MMP-9, TIMP-1 and TIMP-2 in group-I and group-II MM patients

Serum total MMP-2 revealed significant positive (**p=0.002**) correlation with serum TIMP-2. No correlation was observed between serum total MMP-9 and serum TIMP-1 (**table-9**).

Table-9: Correlation between serum total MMP-2 and TIMP-2 as well as MMP-9 and TIMP-1

Parameters	TIMP-2
Total MMP-2	**r =0.579; p=0.002**
	TIMP-1
Total MMP-9	r =-0.063; p=0.753

4.1.8 Isolation of M-Protein and analysis of M-Protein Proteome:

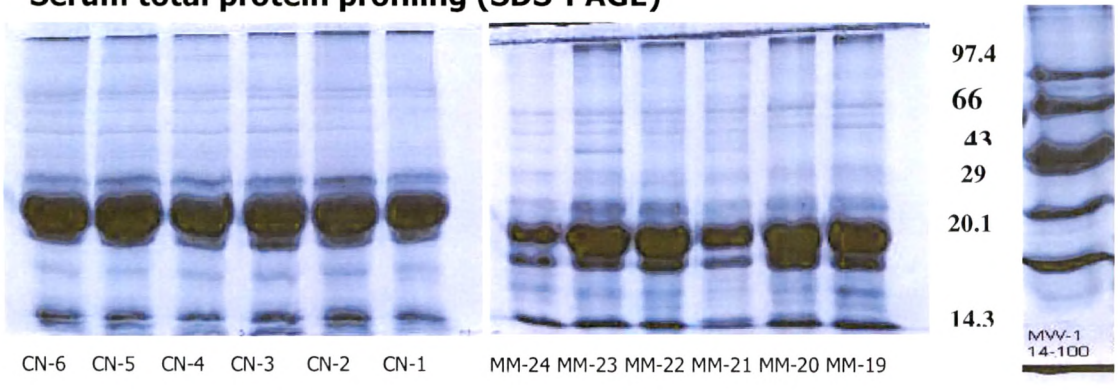
The isolation and study of changes of M-Protein proteome included following steps.

1. Isolation of M-protein

Along with the native-PAGE, serum total protein profiling was also carried-out by SDS-PAGE. SDS breaks S-S bonds of the protein so that after electrophoresis staining and destaining process different subunit of the protein (**figure: 42**) are separated. The M-Protein subunits were also separated because of the SDS treatment.

Figure-42: Representative patterns of serum protein profiling by SDS-PAGE in controls and MM patients

Serum total protein profiling (SDS-PAGE)



2. Molecular weight determination of isolated M-protein

Figure-43: SDS-PAGE patterns of protein molecular weight marker and isolated M-Protein in MM patients

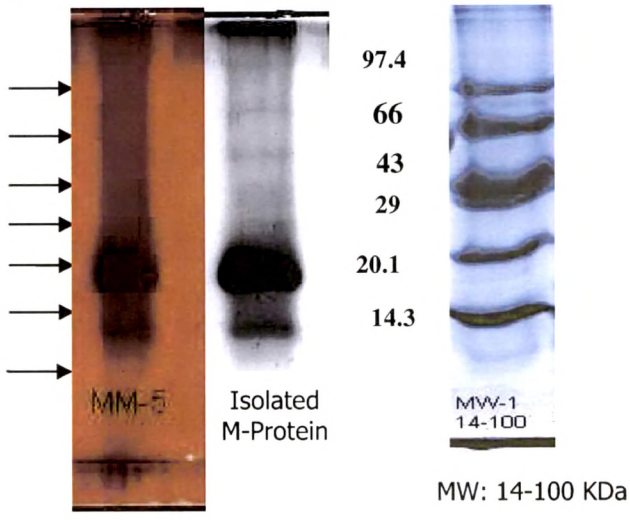
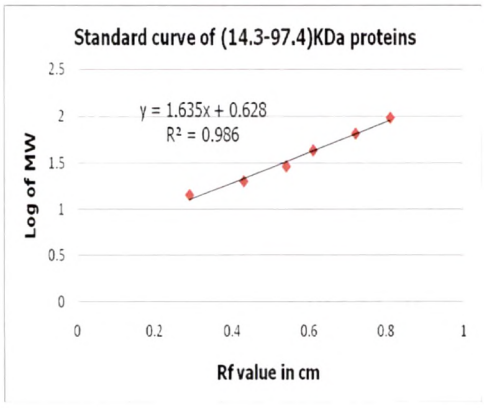


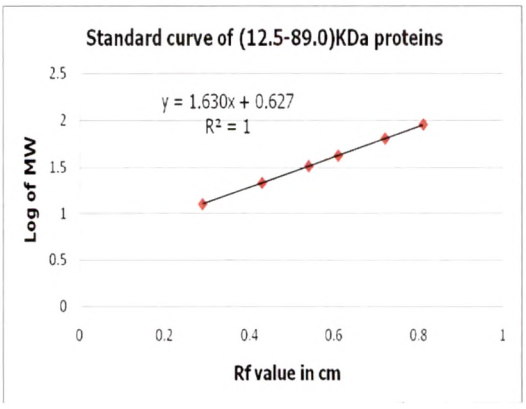
Figure: 43 shows the M-Protein isolated and electro-transfered on nitrocellulose membrane. It was observed that M-protein constituted 7 bands which were separated by SDS-PAGE. The molecular weight of the proteins were further determined with the help of standard protein molecular weight marker.

As documented in **figure: 44** the standard molecular weight marker proteins and calculated proteins curves revealed a linear correlation.

Figure-44: Representative graphs of standard protein and calculated protein molecular weight marker



Standard Molecular weight of Protein



Calculated Molecular weight of protein

Table-10: Comparison of standard protein molecular weight marker and molecular weight of isolated M-Protein

Standard Mol.Wt (Kda) (14-100)	Rf Value	Log Of Mol. Wt	Log of MW of unknown proteins calculated using formula (Y=mx+c)	Converted Log values in normal Mol.Wt (Kda)
14.3	0.29	1.15	1.10	12.5
20.1	0.43	1.30	1.33	21.4
29	0.54	1.46	1.51	32.4
43	0.61	1.63	1.62	41.7
66	0.72	1.81	1.80	63.1
97.4	0.81	1.98	1.95	89.0
Molecular weights of seven bands of isolated M-protein				
-	0.27	1.05	1.06	~11.48
-	0.38	1.25	1.25	~17.78
-	0.47	1.39	1.39	~24.54
-	0.56	1.54	1.54	~34.67
-	0.63	1.66	1.66	~45.70
-	0.74	1.84	1.84	~69.18
-	0.84	2.0	2.00	~100

Table-10 gives comparison of standard protein molecular weight and calculated molecular weight markers of protein. In accordance with seven protein bands were identified from isolated M-protein. The molecular weight of the fractions of isolated M-protein were ~11.48, ~17.78, ~24.54, ~34.67, ~45.70, ~69.18 and ~100; respectively.

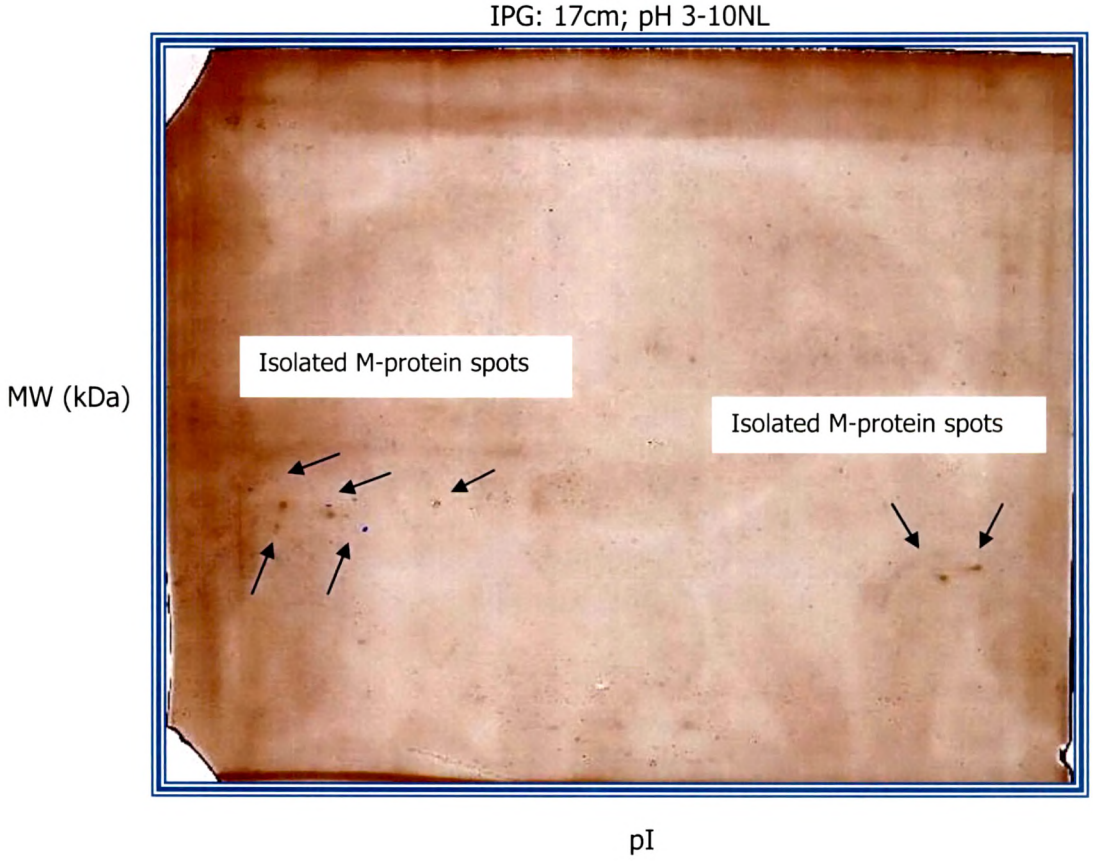
3. 2D-PAGE analysis of M-protein

Study of M-Protein by 2D-PAGE: The 2D-PAGE approaches consisting of isoelectric focusing (1^{st} dimension) and SDS-PAGE (2^{nd} dimension) were attempted to study M-protein. Isolated M-Protein was used as a sample and passive rehydration was carried out. The IPG strips of various pH gradients and different lengths were used for standardization. The most appropriate

separation was obtained with 17 cm; pH 3-10NL IPG strip. The separation was superior when compared with IPG strips of other pH range and lengths. Iso-electro focusing was carried out in 3 steps with final focusing at 40,000 Volts/hour. It was followed by SDS-PAGE which revealed distinct separation of M-protein spots. The gel was stained with silver staining and destained until clear spot were obtained. 2D maps revealed seven distinct protein spots. The spots were observed in the pH range of 3-5 and pH range 8-10.

Figure: 45 shows 2D-PAGE maps of isolated M-protein, which were scanned using gel documentation system and the digitalized images were studied using PDQuest, the discovery series™ software.

Figure-45: Representative patterns of isolated M-Protein by 2D-PAGE



4. Comparison of glycosylation status between group-I and group-II MM patients

Figure-46: Levels of glycoconjugates in isolated M-protein

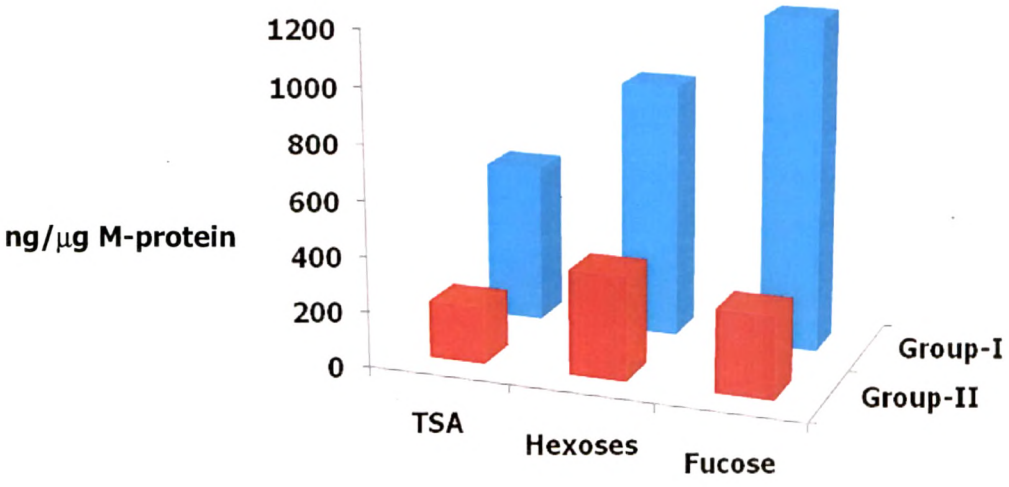


Figure: 46 shows that levels of glycoconjugates (TSA, hexoses and fucose) increased in group-I as compared to group-II MM patients, which indicated higher glycosylation in group-I MM patients.

4.2 CERVICAL CANCER PATIENTS

4.2.1 Total protein profiling in cervical cancer patients

Figure-47: Representative native-PAGE patterns in controls and cervical cancer patients

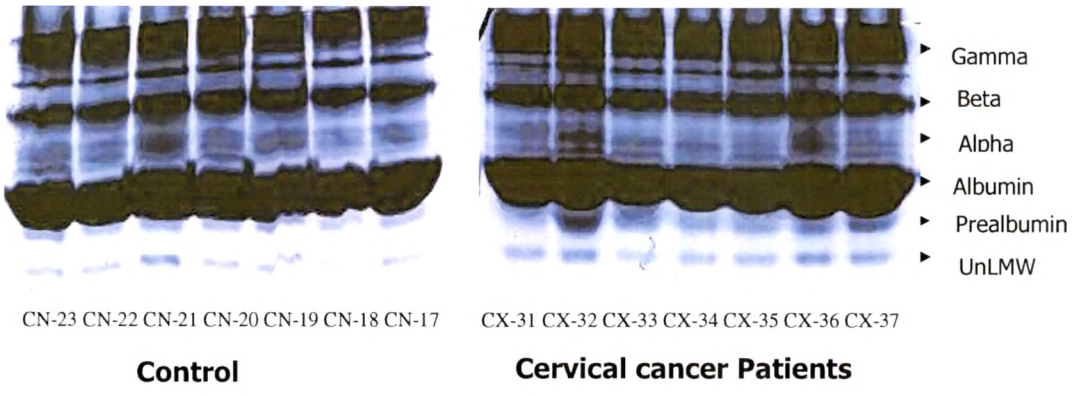
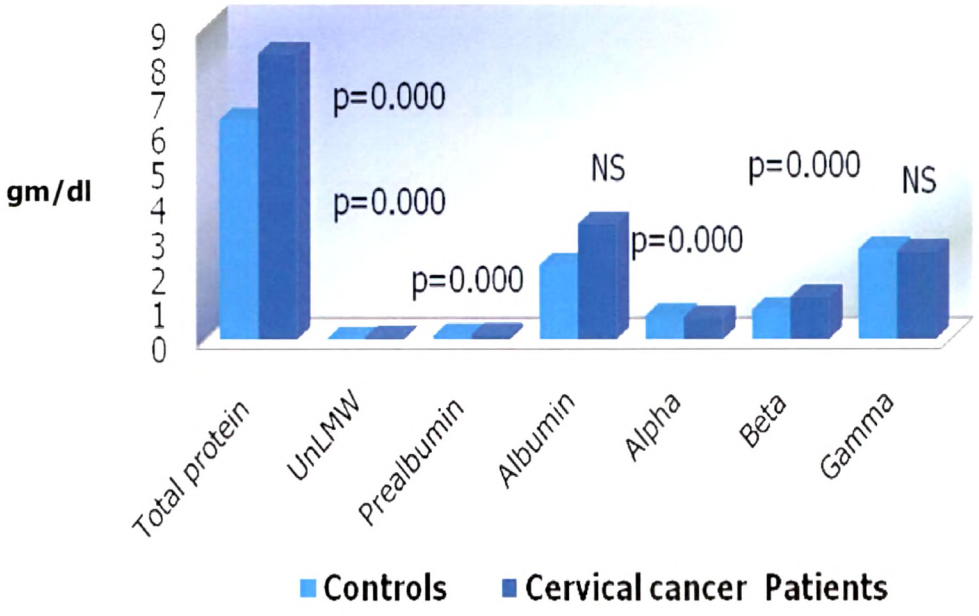


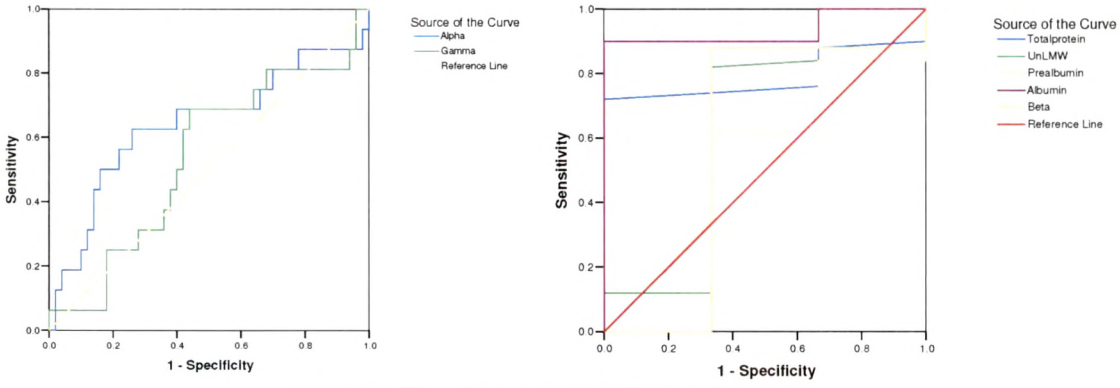
Figure: 47 shows representative patterns of the serum total protein profiles in normal and disease state i.e. controls and cervical cancer patients.

Figure-48: Comparison of the serum native-PAGE profiles between controls and cervical cancer patients



Serum total protein levels, UnLMW, prealbumin, alpha and beta fractions were found to be significantly elevated (**p=0.000, p=0.000, p=0.000 p=0.000** and **p=0.000;** respectively) in cervical cancer patients as compared to the controls. Whereas serum albumin and gamma fractions were comparable between the two groups of the subjects (**figure: 48**).

Figure-49: ROC curve for comparison between controls and cervical cancer patients



Parameters	AUC	Significance
Total protein	0.790	p=0.094
UnLMW	0.597	p=0.577
Prealbumin	0.487	p=0.939
Albumin	0.933	**p=0.012**
Alpha	0.641	p=0.091
Beta	0.587	p=0.617
Gamma	0.536	p=0.664

*AUC: Area under curve

Serum albumin (**p=0.012, AUC=0.933**) could significantly discriminate between the parameters in serum total protein profiles by native-PAGE electrophoresis between controls and cervical cancer patients (**figure: 49**).

Multivariate analysis with clinicopatological parameters:

Multivariate analysis was carried out to correlate variations in serum total protein and glycoprotein profiling, serum total protein and glycoprotein profiles ratio, serum glycoconjugates, protein ratio of glycoconjugates, MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2 with clinicopathological parameters including age, histopathology, stage of the disease and pathological tumour differentiation in the cervical cancer patients.

Table-11: Multivariate analysis between serum protein profiles (native-PAGE) and clinicopathological parameters in cervical cancer patients

Parameters	Age	Histopathology	Early vs. Advanced stage	Pathological tumour differentiation
Total protein	F=1.127 p=0.374	F=0.007 p=0.936	F=0.951 p=0.334	F=2.034 p=0.145
UnLMW	F=1.547 p=0.139	F=0.493 p=0.487	F=0.533 p=0.469	F=1.173 p=0.321
Prealbumin	F=0.682 p=0.802	F=1.793 p=0.188	**F=5.797** **p=0.020**	F=0.606 p=0.551
Albumin	F=1.478 p=0.165	F=1.036 p=0.315	F=0.622 p=0.434	F=1.305 p=0.283
Alpha	F=0.501 p=0.937	F=0.842 p=0.364	F=0.897 p=0.348	F=1.601 p=0.215
Beta	F=0.744 p=0.742	F=2.855 p=0.099	F=0.000 p=0.995	F=0.515 p=0.602
Gamma	F=1.711 p=0.092	F=0.430 p=0.516	F=0.248 p=0.621	**F=5.404** **p=0.009**

The multivariate analysis for variations in serum protein profiles fractions including total protein, UnLMW, prealbumin, albumin, alpha, beta, gamma and various clinicopathological parameters showed that the alterations in prealbumin was significantly (**F=5.797, p=0.020**) associated with early vs. advanced stage of the disease. The alterations in gamma fractions was significantly (**F=5.404, p=0.009**) associated with pathological tumour

differentiation. Remaining fractions could not exhibit any significant correlation with the clinicopathologocal parameters (**table-11**).

Figure: 50 shows comparison between serum total protein profile ratio in controls and cervical cancer patients. Serum total protein, UnLMW:gamma, prealbumin:gamma, albumin:gamma, alpha:gamma and beta:gamma were significantly elevated (**p=0.000, p=0.000, p=0.000, p=0.000, p=0.000** and **p=0.000;** respectively) in cervical cancer patients as compared to the controls.

Figure-50: Comparison of serum total protein profile ratio by native-PAGE between controls and cervical cancer patients

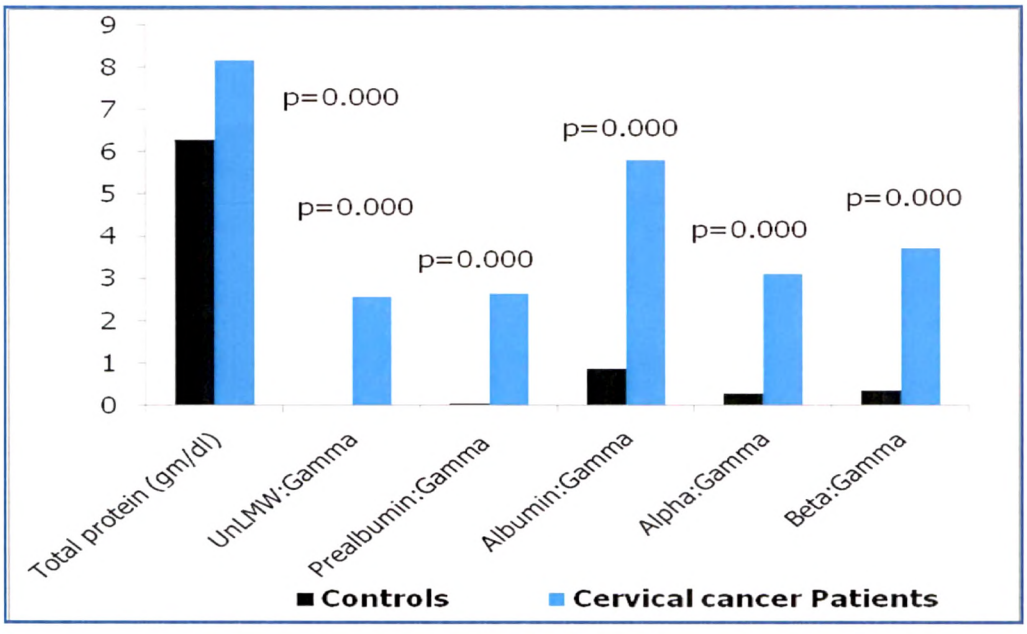
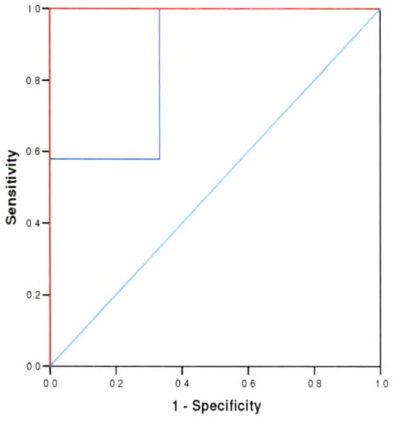
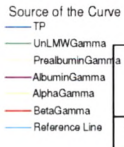


Figure-51: ROC curve for comparison between controls and cervical cancer patients





Parameters	AUC	Significance
Total protein	0.860	**p=0.038**
UnLMW:gamma	1.000	**p=0.004**
Prealbumin:gamma	1.000	**p=0.004**
Albumin:gamma	1.000	**p=0.004**
Alpha:gamma	1.000	**p=0.004**
Beta:gamma	1.000	**p=0.004**

*AUC: Area under curve

As evident from **figure: 51**, serum total protein, UnLMW:gamma, prealbumin:gamma, albumin:gamma, alpha:gamma and beta:gamma could significantly (**p=0.038, AUC=0.860**; **p=0.004, AUC=1.000**; **p=0.004, AUC=1.000**; **p=0.004, AUC=1.000**; **p=0.004, AUC=1.000**; **p=0.004** and **AUC=1.000;** respectively) discriminate between controls and cervical cancer patients.

Table-12: Multivariate analysis between serum protein profiles ratio and clinicopathological parameters in cervical cancer patients

Parameters	Age	Histo-pathology	Early vs. Advanced stage	Pathological tumour differentiation
Total protein	F=1.127 p=0.374	F=0.007 p=0.936	F=0.951 p=0.334	F=1.654 p=0.194
UnLMW:gamma	F=1.722 p=0.089	F=0.482 p=0.492	F=0.294 p=0.590	**F=5.958** **p=0.002**
Prealbumin:gamma	F=1.420 p=0.191	F=0.200 p=0.657	F=0.671 p=0.417	**F=5.793** **p=0.002**
Albumin:gamma	F=1.583 p=0.127	F=0.908 p=0.346	F=0.077 p=0.782	F=2.577 p=0.068
Alpha:gamma	F=1.240 p=0.291	F=0.118 p=0.733	F=0.471 p=0.496	**F=6.111** **p=0.002**
Beta:gamma	F=1.336 p=0.233	F=0.004 p=0.948	F=0.152 p=0.698	**F=4.682** **p=0.007**

Table-12 represents multivariate analysis of serum protein profiles ratio and clinicopathological parameters. Multivariate analysis between total protein, UnLMW:gamma, prealbumin:gamma, albumin:gamma, alpha:gamma and beta:gamma and various clinicopathological parameters revealed that the alterations in UnLMW:gamma, prealbumin:gamma, alpha:gamma and beta:gamma exhibited significant positive (**F=5.958 p=0.002, F=5.793 p=0.002, F=6.111 p=0.002** and **F=4.682 p=0.007**; respectively) association with pathological tumour differentiation.

Figure-52: Representative patterns of serum protein profiles by SDS-PAGE in controls and cervical cancer patients

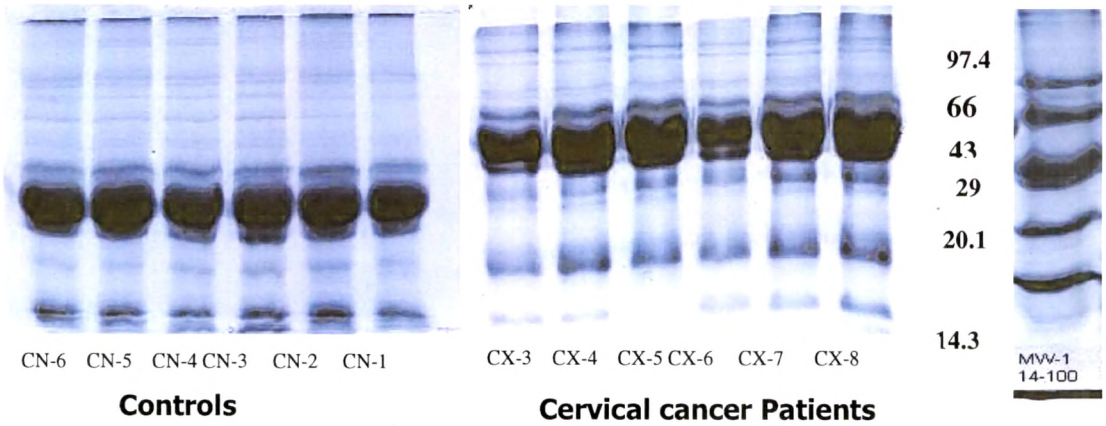


Figure-52 represents different subunits separated by the SDS-PAGE from serum samples. Molecular weight marker revealed that the molecular weight of the fractions ranged between 14 KDa and 100 KDa.

4.2.2 Study of glycosylation changes in cervical cancer

Figure-53: Comparison of serum glycoconjugates and protein ratio of glycoconjugates between controls and cervical cancer patients

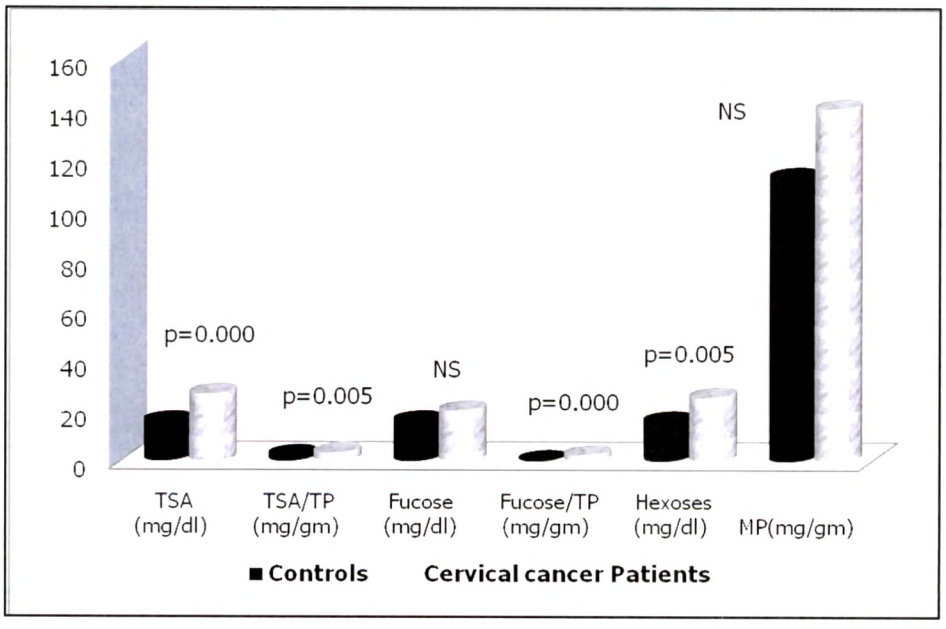


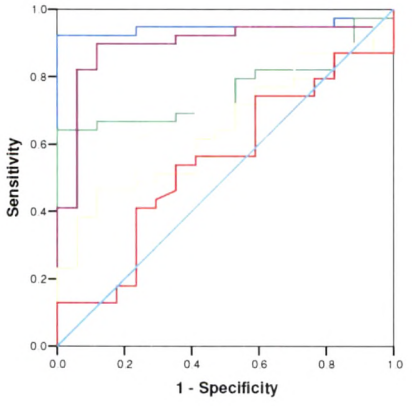
Figure: 53 shows comparison of serum glycoconjugates and protein ratio of glycoconjugate levels between controls and cervical cancer patients. Serum

TSA, TSA/TP, fucose/TP, and hexoses levels were significantly elevated (**p=0.000, p=0.005, p=0.000** and **p=0.005**; respectively) in cervical cancer patients as compared to the controls. Mean values of MP and fucose were higher in the cervical cancer patients, however, the difference was not significant.

ROC Analysis:

As evident from **figure: 54**, serum TSA, TSA/TP, fucose and fucose/TP could significantly (**p=0.000, AUC=0.947; p=0.002, AUC=0.762; p=0.032, AUC=0.681** and **p=0.000 AUC=0.893**; respectively) discriminate between controls and cervical cancer patients.

Figure-54: ROC curve for comparison between controls and cervical cancer patients



Parameters	AUC	Significance
TSA	0.947	**p=0.000**
TSA/TP	0.762	**p=0.002**
Fucose	0.681	**p=0.032**
Fucose/TP	0.893	**p=0.000**
Hexoses	0.638	p=0.103
MP	0.547	p=0.581

*AUC: Area under curve

Correlation between serum glycoconjugates in cervical cancer patients

Table-13: Correlation between glycoconjugates and protein ratio of glycoconjugates

Parameters	TSA/TP
TSA	**r =0.898; p=0.000**
	Fucose/TP
Fucose	**r =0.853; p=0.000**
	MP
Hexoses	**r =0.836; p=0.000**

Correlation coefficients were calculated for serum levels of glycoprotein constituents in cervical cancer patients (**table-13**). Serum TSA, fucose and hexoses showed significant positive correlation (**p=0.000, p=0.000** and **p=0.000;** respectively) with serum TSA/TP, fucose/TP and MP, respectively.

Table-14 represents multivariate analysis for variations in glycoconjugates, protein ratio of glycoconjugates and clinicopathological parameters. Multivariate analysis of serum TSA, TSA/TP, fucose, fucose/TP, hexoses and MP with various clinicopathological parameters showed that the alterations in hexoses and MP were significantly (**F=4.551 p=0.047** and **F=5.221 p=0.028**; respectively) associated with early vs. advanced stage of the disease.

Table-14: Multivariate analysis between glycoconjugates, protein ratio of glycoconjugates and clinicopathological parameters in cervical cancer patients

Parameters	Age	Histopathology	Early vs. Advanced stage	Pathological tumour differentiation
TSA	F=0.487 p=0.933	F=0.021 p=0.885	F=0.178 p=0.675	F=2.033 p=0.148
TSA/TP	F=0.437 p=0.957	F=0.256 p=0.616	F=0.084 p=0.774	F=1.337 p=0.277
Fucose	F=0.382 p=0.976	F=0.086 p=0.771	F=0.928 p=0.342	F=0.174 p=0.841
Fucose/TP	F=0.426 p=0.961	F=0.298 p=0.589	F=1.847 p=0.182	F=1.079 p=0.352
Hexoses	F=0.663 p=0.804	F=0.610 p=0.440	**F=4.551 p=0.047**	F=1.901 p=0.166
MP	F=0.445 p=0.953	F=0.039 p=0.844	**F=5.221 p=0.028**	F=0.985 p=0.384

4.2.3 Study of glycoprotein profiling in cervical cancer patients

Figure-55 shows glycosylation patterns of prealbumin, albumin, alpha, beta and gamma glycoprotein regions in controls and cervical cancer patients separated by native-PAGE.

Figure-55: Representative patterns of glycoproteins by native-PAGE in controls and cervical cancer patients

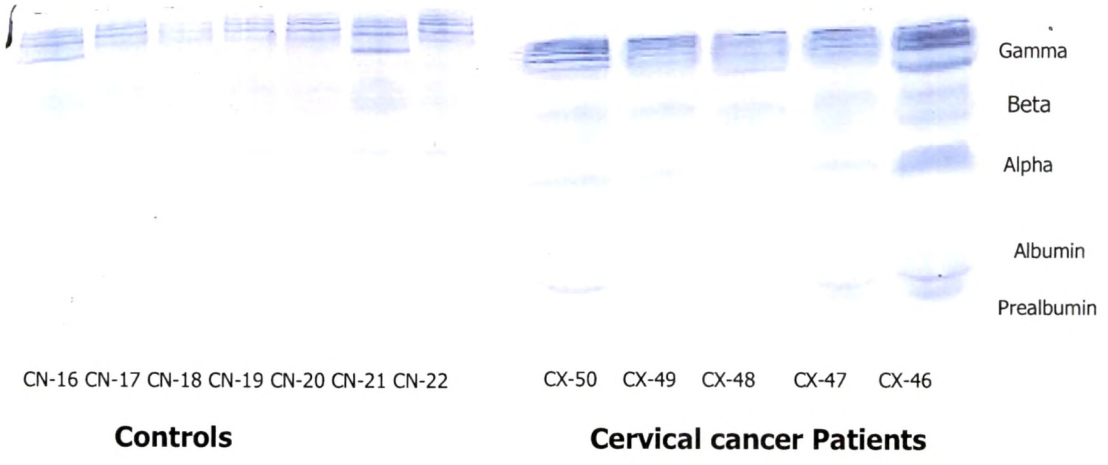
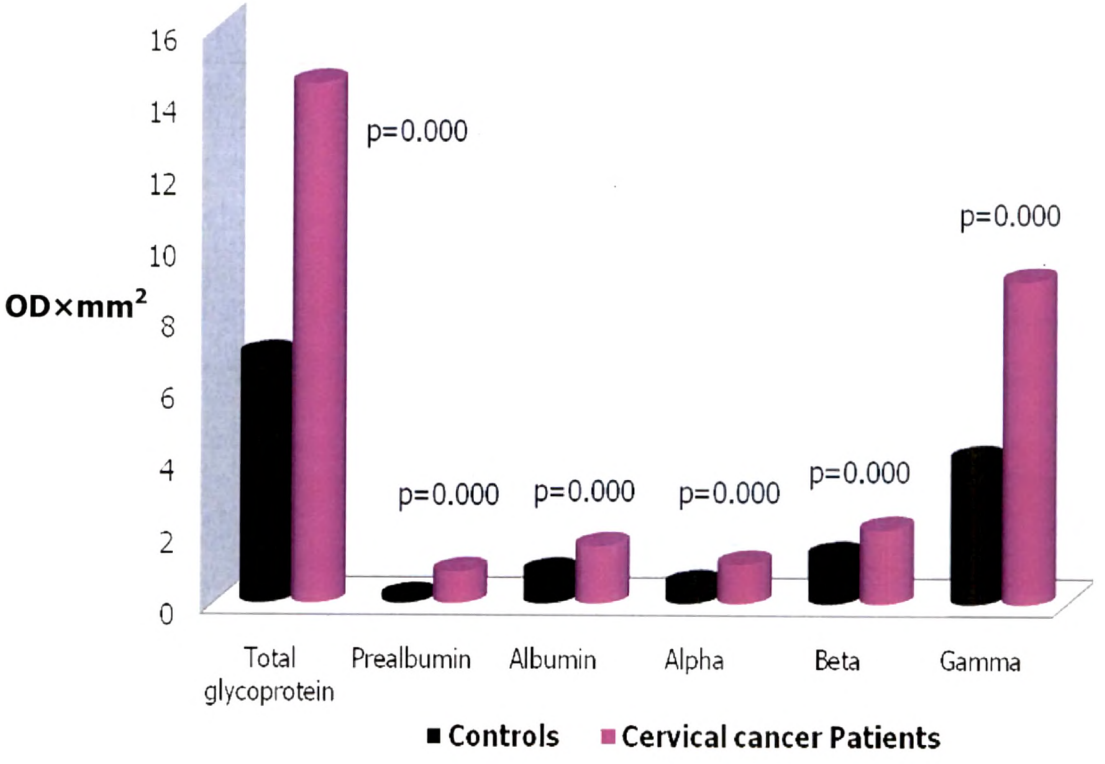
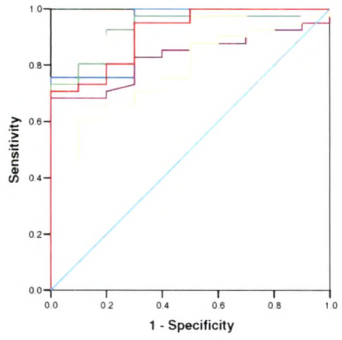


Figure-56: Comparison of glycoprotein native-PAGE profiles between controls and cervical cancer patients



Serum total glycoprotein, prealbumin, albumin, alpha, beta and gamma glycoproteins were found to be significantly higher (**p=0.000, p=0.000, p=0.000, p=0.000, p=0.000, p=0.000** and **p=0.000**; respectively) in cervical cancer patients as compared to the controls (**figure: 56**).

Figure-57: ROC curve for comparison of glycoprotein profiles between controls and cervical cancer patients



Parameters	AUC	Significance
Total glycoprotein	0.927	**p=0.000**
Prealbumin	0.929	**p=0.000**
Albumin	0.905	**p=0.000**
Alpha	0.833	**p=0.001**
Beta	0.783	**p=0.006**
Gamma	0.915	**p=0.000**

*AUC: Area under curve

As evident from **figure: 57,** serum total glycoprotein, prealbumin, albumin, alpha, beta and gamma could significantly (**p=0.000, AUC=0.927; p=0.000, AUC=0.929; p=0.000, AUC=0.905; p=0.001, AUC=0.833; p=0.006, AUC=0.783** and **p=0.000, AUC=0.915**; respectively) discriminate between controls and cervical cancer patients.

Table-15: Multivariate analysis between serum glycoprotein profiles and clinicopathological parameters in cervical cancer patients

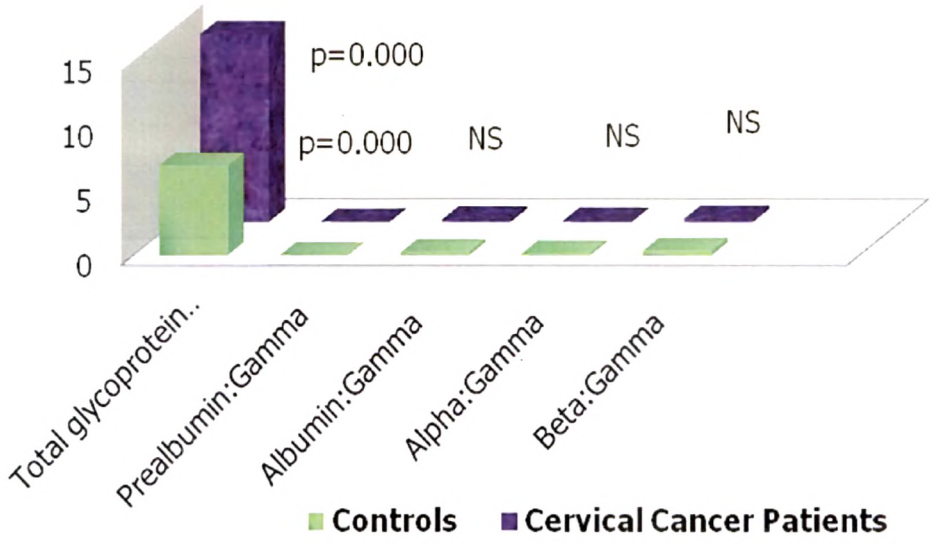
Parameters	Age	Histopathology	Early vs. Advanced stage	Pathological tumour differentiation
Total glycoprotein	F=0.692 p=0.769	**F=4.119** **p=0.049**	F=0.149 p=0.701	F=2.201 p=0.104
Prealbumin	F=0.463 p=0.938	F=1.090 p=0.303	F=1.185 p=0.282	F=0.611 p=0.612
Albumin	F=0.834 p=0.636	F=0.378 p=0.542	F=0.645 p=0.426	F=2.487 p=0.076
Alpha	F=1.381 p=0.231	F=0.943 p=0.337	F=1.070 p=0.306	F=1.873 p=0.151
Beta	F=1.471 p=0.191	F=2.472 p=0.124	F=0.384 p=0.539	F=0.245 p=0.864
Gamma	F=0.695 p=0.766	**F=5.028** **p=0.030**	F=0.154 p=0.697	F=2.668 p=0.062

Table-15 represents multivariate analysis of serum glycoprotein profiles and clinicopathological parameters. Multivariate analysis of total glycoprotein, prealbumin, albumin, alpha, beta, gamma and various clinicopathological parameters showed that the alterations in total glycoprotein and gamma were

significantly (**F=4.119 p=0.049** and **F=5.028 p=0.030**; respectively) associated with histopathology.

Serum total glycoprotein and prealbumin:gamma were found to be significantly elevated (**p=0.000** and **p=0.000**; respectively) in cervical cancer patients as compared to the controls. Other parameters were found to be not significantly altered (**figure: 58**).

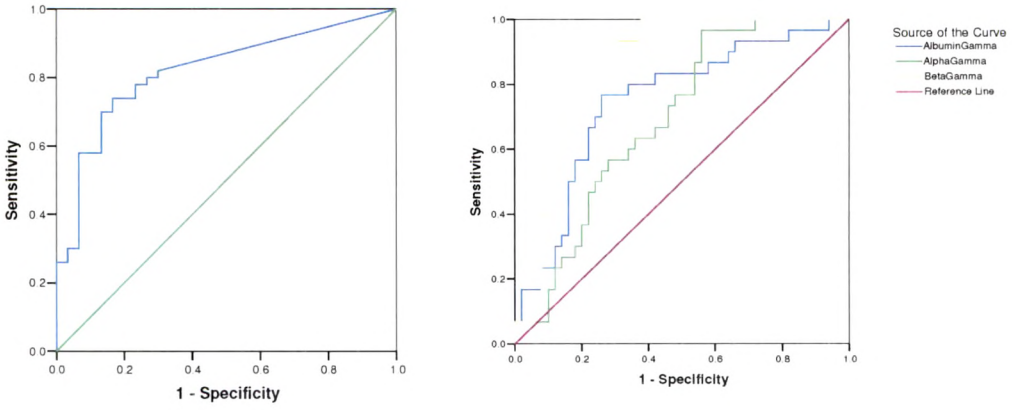
Figure-58: Comparison of glycoprotein profiles ratio by native-PAGE between controls and cervical cancer patients



ROC Analysis:

ROC curve analyses were performed for serum glycoprotein profiles ratio by native-PAGE electrophoresis between controls and cervical cancer patients. As apparent from **figure: 59**, serum prealbumin:gamma, albumin:gamma, alpha:gamma and beta:gamma could significantly (**p=0.000, AUC=0.820, p=0.000, AUC=0.747, p=0.004, AUC=0.692** and **p=0.000, AUC=0.875**; respectively) discriminate between controls and cervical cancer patients.

Figure-59: ROC curve for comparison of native-PAGE glycoprotein profiles between controls and cervical cancer patients



Parameters	AUC	Significance
Prealbumin:gamma	0.820	**p=0.000**
Albumin:gamma	0.747	**p=0.000**
Alpha:gamma	0.692	**p=0.004**
Beta:gamma	0.875	**p=0.000**

*AUC: Area under curve

Table-16: Multivariate analysis between serum glycoprotein profiles ratio and clinicopathological parameters in cervical cancer patients

Parameters	Age	Histo-pathology	Early vs. Advanced stage	Pathological tumour differentiation
Total glycoprotein	F=0.686 p=0.798	**F=4.119** **p=0.049**	F=0.149 p=0.701	F=2.201 p=0.104
Prealbumin:gamma	F=1.203 p=0.317	F=1.904 p=0.175	F=2.100 p=0.154	F=0.571 p=0.637
Albumin:gamma	F=0.883 p=0.600	**F=25.89** **p=0.000**	F=2.207 p=0.144	F=0.294 p=0.829
Alpha:gamma	F=1.319 p=0.243	**F=11.177** **p=0.002**	F=0.001 p=0.977	F=0.063 p=0.979
Beta:gamma	F=0.832 p=0.653	F=1.558 p=0.219	**F=4.202** **p=0.046**	F=0.708 p=0.553

Multivariate analysis of serum total glycoprotein, prealbumin:gamma, albumin:gamma, alpha:gamma and beta:gamma and various clinicopathological parameters showed that the alterations in total glycoprotein, albumin:gamma and alpha:gamma were found to be significantly associated with histopathology (**F=4.119 p=0.049, F=25.89 p=0.000** and **F=11.17 p=0.002**; respectively) and beta:gamma was found

significantly (**F=4.202, p=0.046**) associated with early vs advanced stage of the disease (**table-16**).

4.2.4 Expression of MMPs in cervical cancer patients

Figure-60: Representative zymograms of MMP-2 and MMP-9 in controls and cervical cancer patients

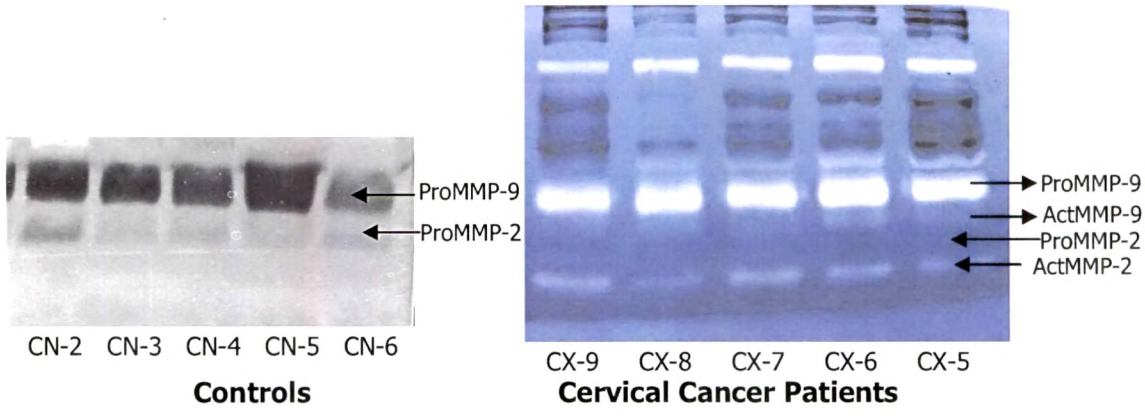
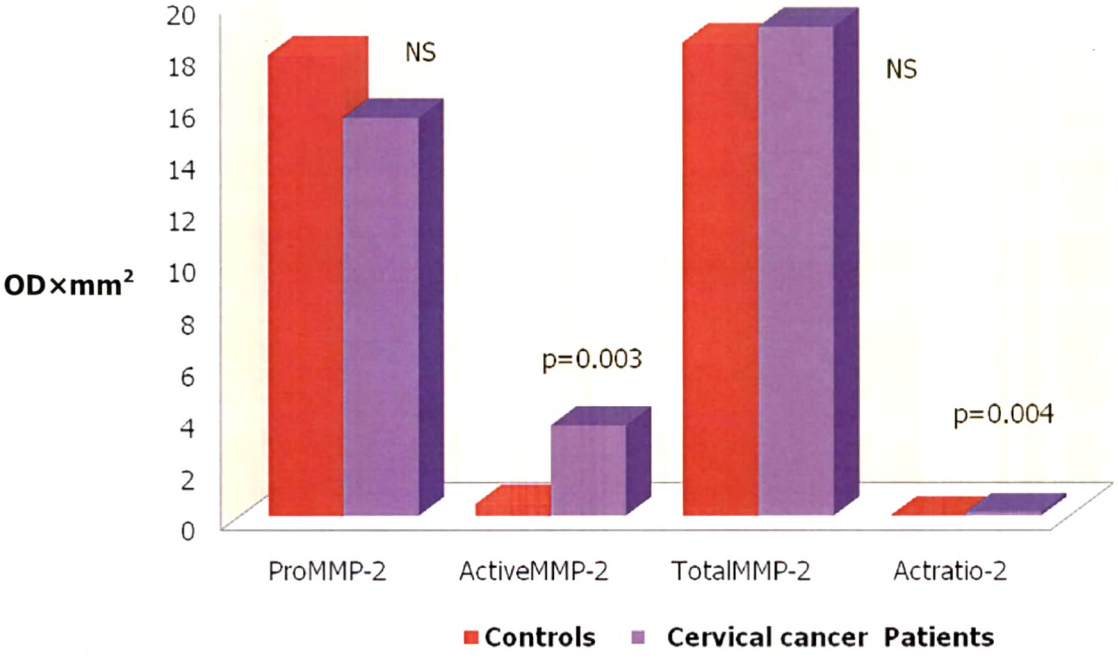
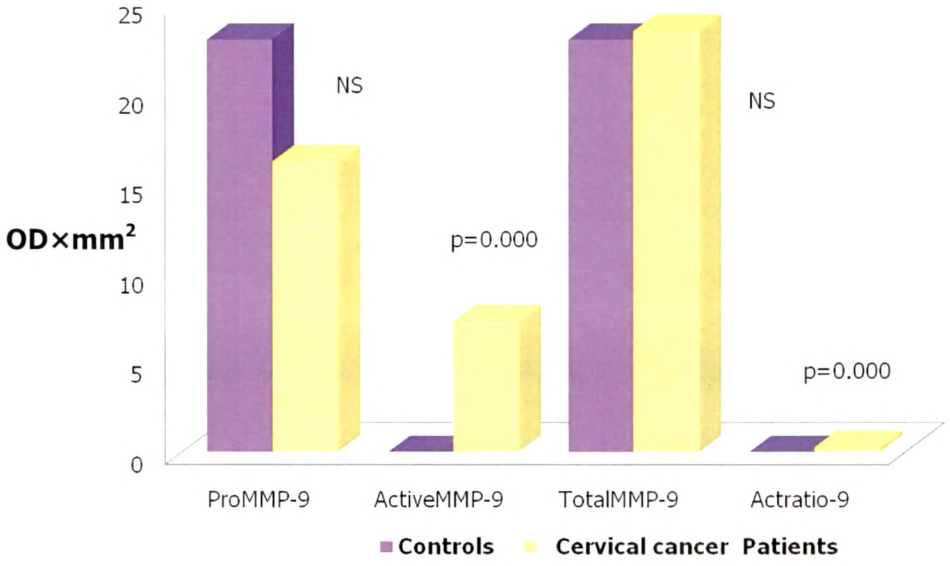


Figure-61: Comparison of serum proMMP-2, activeMMP-2, totalMMP-2 and activation ratio of MMP-2 by zymography between controls and cervical cancer patients



Serum activeMMP-2 and activation ratio of MMP-2 were significantly higher (**p=0.003** and **p=0.004**; respectively) in cervical cancer patients as compared to the controls (**figure: 61**).

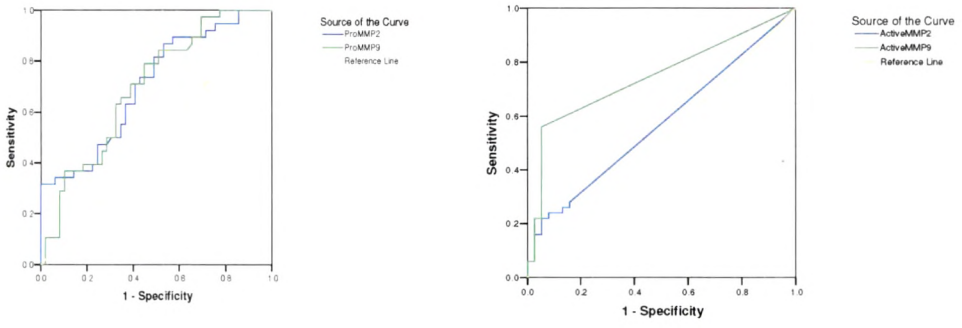
Figure-62: Comparison of serum proMMP-9, activeMMP-9, totalMMP-9 and activation ratio of MMP-9 by zymography between controls and cervical cancer patients



As shown in **figure: 62,** serum active MMP-9 and activation ratio of MMP-9 were significantly higher (**p=0.000** and **p=0.000;** respectively) in cervical cancer patients as compared to the controls.

ROC Analysis:

Figure-63: ROC curve for comparison between controls and cervical cancer patients



Parameters	AUC	Significance
ProMMP-2	0.705	**p=0.001**
ActiveMMP-2	0.573	P=0.244
ProMMP-9	0.665	**p=0.002**
ActiveMMP-9	0.752	**P=0.000**

*AUC: Area under curve

As evident from **figure: 63**, serum proMMP-2, proMMP-9 and activeMMP-9 could significantly (**p=0.001, AUC=0.705**; **p=0.002, AUC=0.665** and

p=0.000, AUC=0.752; respeectively) discriminate between controls and cervical cancer patients.

Correlation of serum pro and active forms of MMP-2 and MMP-9 by zymography analysis in cervical cancer patients

As documented in **table-17,** serum proMMP-2 were negatively and significantly (**p=0.000**) as well as positively and significantly (**p=0.000**) correlated with serum activeMMP-2 and serum total MMP-2, respectively. Serum proMMP-9 showed significant positive (**p=0.000**) association with total MMP-9. No correlation was found between proMMP-9 and activeMMP-9.

Table-17: Correlation between serum pro, active and total forms of MMP-2 and MMP-9

Parameters	ActiveMMP-2	TotalMMP-2
Pro MMP-2	**r = -0.445; p=0.000**	**r = 0.723; p=0.000**
	ActiveMMP-9	TotalMMP-9
Pro MMP-9	r = -0.147; p=0.229	**r = 0.889; p=0.000**

Table-18: Correlation between serum pro, active and total forms of MMP-2 and MMP-9

Parameters	ProMMP-9	ActiveMMP-9	Total MMP-9
Pro MMP-2	**r = -0.445** **p= 0.000**	r = -0.015 p= 0.903	**r= 0.530;** **p= 0.000**
	ProMMP-2	ActiveMMP-2	Total MMP-2
Pro MMP-9	**r = 0.561** **p= 0.000**	**r = -0.262** **p= 0.029**	**r = 0.396** **p= 0.001**

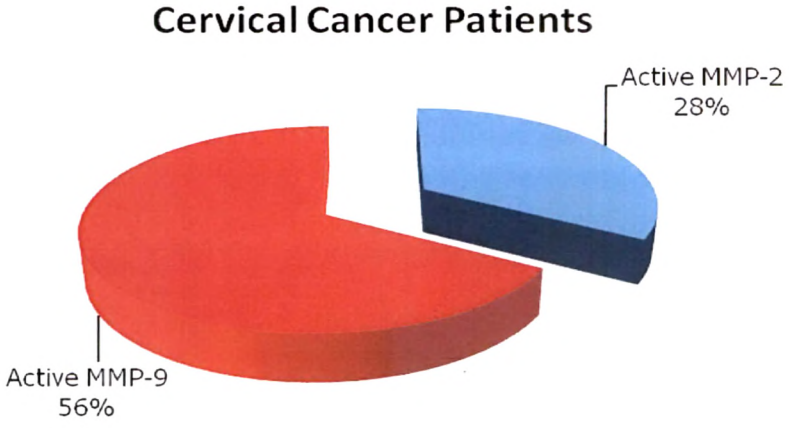
Serum proMMP-2 were negatively and significantly (**p=0.000**) as well as positively and significantly (**p=0.000**) correlated with serum proMMP-9 and serum total MMP-9, respectively. Serum proMMP-9 were found to be positively and significantly (**p=0.000** and **p=0.001;** respectively) correlated with serum proMMP-2 and total MMP-2. Serum proMMP-9 was found to be negatively and significantly (**p=0.029**) associated with serum activeMMP-2 (**table-18**).

Table-19: Correlation between active forms of MMP-2 and MMP-9

Parameter	ActiveMMP-9
ActiveMMP-2	**r = -0.234; P=0.05**

Pearson's correlation coefficients showed that serum activeMMP-2 was found to be negatively and significantly (**P=0.05**) correlated with serum activeMMP-9 (**table-19**).

Figure-64: Percentage activity of active forms of MMP-2 and MMP-9 in cervical cancer patients



The active forms of MMP-2 and MMP-9 were found activation in 28% and 56% in cervical cancer patients (**figure-64**).

Table-20: Multivariate analysis between serum MMP-2 and clinicopathological parameters in cervical cancer patients

Parameters	**Age**	**Histo-pathology**	**Early vs. Advanced stage**	**Pathological tumour differentiation**
Pro MMP-2	F=1.258 p=0.280	F=0.778 p=0.383	F=0.523 p=0.473	F=0.948 p=0.427
Active MMP-2	F=1.505 p=0.155	F=0.039 p=0.844	F=0.252 p=0.618	F=0.155 p=0.926
Total MMP-2	F=0.978 p=0.506	F=0.539 p=0.467	F=0.093 p=0.761	F=0.544 p=0.655
Activation ratio MMP-2	**F=2.153 p=0.029**	F=0.089 p=0.767	F=0.383 p=0.539	F=0.122 p=0.947

Multivariate analysis of serum pro, active, total forms and activation ratio of MMP-2 and various clinicopathological parameters showed that the alterations in activation ratio of MMP-2 were significantly (**F=2.153, p=0.029**) associated with age of the cervical cancer patients (**table-20**).

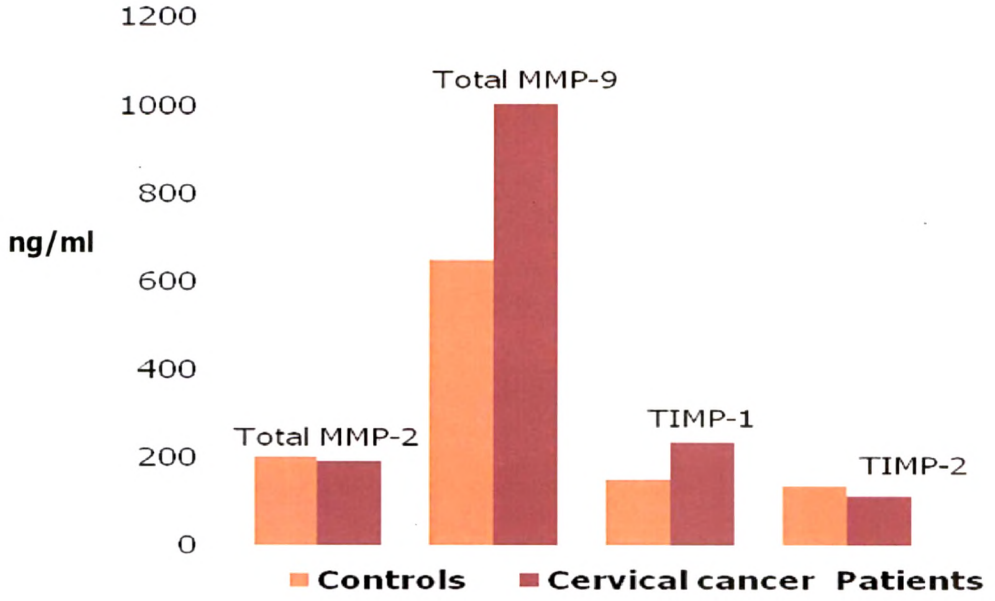
Table-21: Multivariate analysis between serum MMP-9 and clinicopathological parameters in cervical cancer patients

Parameters	Age	Histo-pathology	Early vs. Advanced stage	Pathological tumour differentiation
Pro MMP-9	F=0.265 p=0.998	F=0.000 p=0.985	F=3.192 p=0.081	**F=8.407** **p=0.000**
Active MMP-9	F=1.171 p=0.340	F=3.211 p=0.081	F=0.707 p=0.405	F=1.057 p=0.379
Total MMP-9	F=0.476 p=0.950	F=0.893 p=0.350	**F=4.319** **p=0.043**	**F=4.003** **p=0.015**
Activation ratio MMP-9	F=0.771 p=0.715	F=1.886 p=0.177	F =0.139 p=0.711	F=1.138 p=0.347

Table-21 represents multivariate analysis of serum pro, active, total forms and activation ratio of MMP-9 and various clinicopathological parameters. The analysis showed that the alterations in proMMP-9 and total MMP-9 were significantly (**F=8.407 p=0.000** and **F=4.003 p=0.015;** respectively) associated with pathological tumour differentiation. Total MMP-9 was found significantly (**F=4.319 p=0.043**) associated with early vs. advanced stage of the disease.

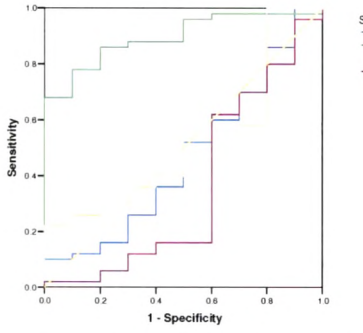
Figure: 65 shows comparison of serum total MMP-2, total MMP-9, TIMP-1 and TIMP-2 levels by ELISA between controls and cervical cancer patients. Serum total MMP-9 values were significantly higher (**p=0.009**) in cervical cancer patients as compared to the controls. Serum TIMP-1 was also found higher level, whereas serum total MMP-2 and TIMP-2 were found to be comparable between controls and cervical cancer patients.

Figure-65: Comparison of serum levels of total MMP-2, total MMP-9, TIMP-1 and TIMP-2 by ELISA between controls and cervical cancer patients



ROC Analysis:

Figure-66: Comparison of serum levels of the total MMP-2, total MMP-9, TIMP-1 and TIMP-2 by ELISA between controls and cervical cancer patients



Parameters	AUC	Significance
Total MMP-2	0.468	p=0.751
Total MMP-9	0.896	**p=0.000**
TIMP-1	0.522	p=0.827
TIMP-2	0.362	p=0.171

*AUC: Area under curve

As evident from **figure: 66,** serum total MMP-9 could significantly (**p=0.000, AUC=0.896**) discriminate between controls and cervical cancer patients.

Correlation between serum total MMP-2, total MMP-9, TIMP-1 and TIMP-2 in cervical cancer patients

Pearson's correlation analysis revealed that serum total MMP-2 showed significant (**p=0.000**) correlation with serum TIMP-2 (**table-22**).

Table-22: Correlation between serum total MMP-2 and TIMP-2 as well as MMP-9 and TIMP-1

Parameters	TIMP-2
Total MMP-2	**r =0.601; p=0.000**
	TIMP-1
Total MMP-9	r =0.213; P=0.126

Multivariate analysis of serum total MMP-2, total MMP-9, TIMP-1, TIMP-2 and various clinicopathological parameters showed that the alterations in serum TIMP-1 was significantly (**F=6.854 p=0.000**) associated with age of the cervival cancer patients. The balance between total protease activity and their inhibitors (MMP-2:TIMP-2) complex was found to be significantly (**F=3.789 p=0.05**) associated with early vs advance stage of the disease (**table-23**).

Table-23: Multivariate analysis of serum total MMP-2, total MMP-9, TIMP-1 and TIMP-2 with clinicopathological parameters in cervical cancer patients

Parameters	**Age**	**Histo-pathology**	**Early vs. Advanced stage**	**Pathological tumour differentiation**
Total MMP-2	F=0.588 p=0.881	F=0.124 p=0.726	F=1.373 p=0.247	F=1.496 p=0.232
Total MMP-9	F=0.844 p=0.640	F=0.568 p=0.455	F=2.359 p=0.131	F=0.499 p=0.685
TIMP-1	**F=6.854 p=0.000**	F=0.122 p=0.729	F=3.313 p=0.075	F=1.443 p=0.246
TIMP-2	F=1.061 p=0.430	F=0.002 p=0.967	F=0.431 p=0.515	F=1.378 p=0.265
MMP-9:TIMP-1	F=1.319 p=0.241	F=0.926 p=0.342	F=0.050 p=0.824	F=1.422 p=0.252
MMP-2:TIMP-2	F=0.228 p=0.999	F=0.224 p=0.638	**F=3.789 p=0.05**	F=2.063 p=0.122