Correlating Mitotic and Nucleolar Organizer Region indices - A better prognosticator of Oral Squamous Cell Carcinoma

Running title AgNOR's prognosticator in oral cancer

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Abstract

Rate of cell proliferation plays a vital role in determining the outcome of oral squamous cell carcinoma (OSCC). Numerous markers exist to assess the proliferation of cell, among which mitotic and nucleolar organizer region (NOR) indices are the most feasible techniques.

Aim: The aim of the study was to correlate the two indices of proliferation with prognosis of OSCC.

Experimental setting and design: Twenty proven cases of OSCC were evaluated for mitotic activity (Mitotic activity index and Volume-corrected mitotic index) and NOR's (number and area) respectively in H&E and silver nitrate stained slides. These parameters were compared with the prognosis (event of recurrence and death) of OSCC.

Statistical analysis: Chi-square, Kruskal wallis test, Bonferroni's Posthoc correction, Spearman's rho and Regression analysis were performed.

Results: All the parameters showed an increasing value from disease free patients to the patient exhibiting recurrence and mortality. The proliferative indices had good to excellent correlation (rho ranged from 0.526 to 0.885). Regression analysis indicated that the combination of mitotic and NOR area showed better prediction of outcome.

Conclusion: Outcome of the disease can be predicted in light microscope with mitotic and AgNOR's indices at ease.

Key words: Cell Proliferation index, Volume corrected Mitotic index, NOR index.

Introduction

Cell proliferation is not only essential for normal sustenance and growth of a tissue but also plays a major role in tumor growth and proliferation. Proliferation indices provide valuable information on the outcome of tumor which can be assessed by mitotic indices, nucleolar organizer regions (NOR's), immunohistochemical markers (Ki67, Ki-S, PCNA) etc. The simplicity and affordability of evaluating mitotic index in the routine H&E staining makes it the most accessible and useful tool. Two of the modifications of mitotic activity assessment have proved to be of prognostic significance, namely mitotic activity index (the number of mitotic figures in a tissue at a given moment) and volume corrected mitotic index (the number of mitotic figures in a given percentage of tumor tissue).[1,2,3] Nucleolar organizer regions are loops of DNA located at the acrocentric chromosomes in humans which transcribe RNA, and can be stained using colloidal silver technique. The number and size/area of NOR's per cell nucleus correlates well with the cell proliferation rate of oral squamous cell carcinoma. [4, 5]

The unpredictable course and morbidity associated with oral squamous cell carcinoma makes it crucial for us to gain more knowledge on the cell kinetics and its association with prognosis. The purpose of this study was to correlate the mitotic as well as AgNOR indices between each other as well as with the prognostic outcome of oral squamous cell carcinoma (OSCC).

Materials and methods

Selection of patients:

The study group consisted of 20 patients with oral squamous cell carcinoma who were diagnosed, treated and followed from 1999 to 2004. The patients had undergone surgery as their initial and only mode of treatment; with histologically clear excisional margins and a follow-up for a minimum of 36 months.

For the mitosis assessment hematoxylin and eosin (H&E) stained paraffin-embedded sections (five μm thick) from the core of the tumour were used. The histological malignancy grading and mode of invasion were judged according to the method proposed by ***Bryne et al*** [6] and the invasive front grading was tabulated for each case. The assessment was done by two of the authors and the values averaged to eliminate the interobserver variability.

Assessment of mitotic activity

1. Mitotic activity index (MAI) : [2]

Mitotic activity index is expressed as the total number of sharply defined mitoses per 10 high power fields at the invasive front. Counting of mitoses was done at ×400 magnification with a 40x objective (numerical aperture – 0.65 and field diameter 450 μm). Once focused, no further adjustments were made and structures that could be interpreted differently (such as artefacts) were not counted. The existence of hairy protrusions within the amphophillic cytoplasm and lack of nuclear membrane were the criterion for mitotic figures. In contrast, the presence of fire cone figure, a dark line paralleling the margin or the presence of large, dark round spots were not regarded as mitotic figures. If all these criteria did not allow a definite assignment, the figure under discussion was eliminated from the measurement.

2. Volume-corrected mitotic index (M/V index):

The M/V index (M for mitosis and V for volume) was determined as described by ***Haapasalo et al***. [3,7]

$$M/V \text{ index} = \sum MI / \sum Vv$$

MI= Number of mitotic figures in randomly selected microscopic field of neoplastic epithelium from the invasive front.

Vv= volume fraction percent of neoplastic epithelium as assessed by "point counting technique" with a 1000 point square grid overlaid on the tumour image in each field of vision. (Figure 1) In each field, after counting the number of mitoses, points overlying the stroma and epithelium were counted and the percentage of points overlying epithelium was taken to be the volume percent of epithelium.

Silver staining for Nucleolar Organizer Regions

Working solution was freshly prepared by mixing Solution of 50% Silver nitrate solution and Solution of Formic acid and Gelatine in a ratio of 2:1. Deionised water was used as solvent for all solutions. The resultant working solution was used immediately for staining. The staining was performed as per the technique described by Kahn et al. [8]

The number of NORs per nucleus was counted in 200 nuclei using x1000 magnification at the invasive front. Only those areas in the nucleus that appeared as separate black dots were recorded; 2 or more dots closely aggregated were counted as one. (Figure 2) The mean number of NORs per cell (N) was calculated using the formula:

$$\textbf{Mean NORs per cell (N)} = \frac{\sum NOR's}{number\ of\ cells}$$

Slides were scanned under x1000 magnification and the invasive front was identified. The selected area was digitally captured and uploaded for Computer-aided Image Analysis.

AgNORs were clearly visible as black dots and easily discriminated from the surrounding background by grey-value threshold of image analysis.

To minimize nucleolus-biased sampling, the field of the image was reduced to central portion with only 2-5 cells as proposed by ***Ruschoff J et al***. [9] AgNORs area for 200 cells in invasive front were calculated. AgNORs area was estimated using IMAGE J version 1.36b (National Institutes of Health, USA). Mean AgNORs area per nucleus was calculated using the following formula:

$$\textbf{Mean AgNORs area per nucleus} = \frac{\sum Area\ of\ NOR's}{number\ of\ cells}$$

Statistical analysis

1. The TNM staging was correlated with the prognosis factors like recurrence and fatality by Chi square test.
2. Analysis of normality by Shapiro Wilks test showed that the parameters of volume corrected mitotic index and mean AgNOR area had a skewed distribution of data thus non parametric tests were employed for analysis. Individual parameters were correlated with the prognosis using Kruskal wallis test followed by Bonferroni's posthoc analysis.
3. NOR variables and Mitotic parameters were correlated using Spearman's Rho.
4. The efficiency of these parameters were analysed using receiver operating curve characteristics. The parameters were pitted against the outcome variable in forward stepwise linear regression analysis to find the best combined prognosticator.

Results

Twenty patients were included in the study. Their ages ranged from 28 to 71 years (mean of 56.65 years), 14 of them were males, the site of tumour were distributed as Buccal

mucosa (7 cases), alveolus (5 cases), tongue (4 cases), palate (2 cases) and one case each involving lip and retromolar region. TNM staging of the tumours classified majority (65%) of the lesions as stage IV, followed by stage III(30%) and stage II(5%).

On correlating the TNM staging with three prognostic events [disease free (8 cases), recurrence(8 cases) and death(4 cases)] it was found that even in TNM stage IV there was even distribution of patients in the categories of disease free(38 %), recurrence (31 %) and death (31 %) with no statistical significance (Fishers exact 4.474, p=0.315).

Comparison between the prognostic parameters with the variables of histological invasive front grading(IFG), Mean AgNOR's number and area, Mitotic activity index (MAI) and Volume Corrected Mitotic Index (M/V index) using Kruskal Wallis test. The variables showed an increase in the values from disease free group to the group exhibiting recurrence and the group exhibiting mortality. (Table 1, Figure 3) Post hoc analysis indicate that the variation is between the disease free group and either recurrence or group exhibiting death. The parameters failed to differentiate between the recurrent and fatal groups.

Spearman's correlation (table 2) indicated that the parameters correlated with each other ranging from good to excellent. The best correlation was observed between MAI and M/V with spearmans $\rho = 0.885$. Scatter plots indicate positive correlation between the proliferative variables (MAI, M/V index, NOR area and number) and IFG. (Figure 4)

To find out the best prognosticator of the adverse event occurring in OSCC, stepwise linear regression analysis was performed with all the five parameters. The variables deemed significant were the volume corrected mitotic index and the Mean AgNOR's Area in the formula of :

$$\text{Predictor One} = 0.139 + 0.322(\text{M/V index}) + 0.550(\text{Mean AgNOR's Area})$$

Comparison of the sensitivity and specificity of these parameters using ROC curve plots showed that the combined parameter had a higher efficiency in predicting death with

area under the curve of 0.953. Invasive front grading had higher efficiency in predicting the recurrence (area of 0.833). (Table 3, Figure 5)

Discussion

Cell kinetics regulates the turn of events in a tumour tissue to a great extent. The increased tumour cell load leads to tumour migration, invasion and metastasis. The faster tumour growth correlates with the fact that there are more number of cells undergoing division (mitotic phase) at a given point of time. Accurate estimation of cell proliferation indicators can give us hidden information of the tumour growth and thus the outcome. Mitotic indices are the oldest and the easiest to assess among the proliferation indices. We must remain cognizant that the assessment of mitotic activity involves the counting of cells in the state of division and is best identified from the stage where chromatin organization at the equatorial plane of the cell (Metaphase) takes place, to the division of the nucleus and cytoplasm (telophase). Also, the tumour contains numerous cells which are aneuploid and are not in a state of division, thus go unaccounted for in the mitotic indices. During division, these aneuploid cells multiply in uneven numbers. The aneuploid status of tumour cell cannot be identified in routine stains as they mimic any other interphase/prophase nuclei. Thus the true malignant potential of the cells go unrecognized. This hidden architecture of the tumour may be identified by analyzing the Nucleolar Organizing Regions (NOR's) using colloidal silver staining. AgNOR represent the loops of DNA on the five pairs of acrocentric chromosomes. In DNA aneuploid cells the number/area of nucleolar organizing regions would be greater in as compared to the DNA diploid cells at any given moment. [8]

The higher mitotic activity doesn't necessarily mean faster rate of division. Studies have shown that the cancer cells (bronchogenic carcinoma) have a slower cell cycle than the normal cells i.e. they remain in the mitotic phase for longer duration. [5] This alteration is due to the abnormal protein or protein products produced by mutated genes. The cell cycle

regulatory proteins are in turn regulated by the rRNA. Nucleolar Organizer Regions (NOR's) are loops of rDNA in the nucleolus which transcribe for rRNA. Thus, increase in NOR number and area in a given tumour correlates not only with the aneuploid status but also the genetic derangement of the tumour. [8]

The results of our study showed significant differences in the mitotic as well as the NOR parameters between the event of recurrence and mortality. Invasive front grading showed the highest efficiency (83.3%) for the prediction of the event of recurrence, followed by mitotic indices. M/V index has been proven to be the best individual prognosticator in various epithelial tumours including OSCC. [3] This is substantiated in the present study too, where in M/V index had an efficiency of 90.6% to predict Mortality.

The very good positive correlation between the mitotic indices and NOR parameters with invasive front grading (r =0.630 to 0.769) along with their efficiency in predicting outcome, indicated their suitability as a prognostic proliferative marker. The combination of the M/V index and the AgNOR area had an efficiency of 95.3 % to predict mortality. This is due to the fact that the number of cells dividing (M/V index) as well as the number of cells with DNA aneuploidy or genetic derangement (AgNOR) are accounted for by the combination.

Mitosis and AgNOR's can be effectively evaluated on a light microscopic level. Thus, the incorporation of these two parameters in the development of biofunctional staging system is advocated as it would elicit better prognostic information determining the outcome of oral squamous cell carcinoma than the clinic-pathological staging.

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Table 1: Comparison of histological, mitotic and NOR variables with Prognosis using Kruskal Wallis test

Parameter	Groups	Minimum	Maximum	Percentiles			Chi-square	Exact Sig.	Post hoc Bonferroni's correction
				25	Median	75			
IFG	disease free (1)	8	12	9.00	10.00	10.00	13.998	**<0.001**	1,2 and 1,3
	Recurrence(2)	13	18	13.25	15.00	15.75			
	Death (3)	14	16	14.00	14.50	15.75			
Mean AgNORs number	disease free (1)	3.650	6.495	4.09750	4.67500	5.46125	9.346	**.004**	1,2 and 1,3
	Recurrence(2)	4.575	9.750	5.47750	6.86250	8.19750			
	Death (3)	6.225	8.250	6.66375	7.99250	8.18875			
Mean AgNORs area	disease free (1)	1.060	2.920	1.32175	1.37000	1.57250	9.702	**.003**	1,2
	Recurrence(2)	1.580	2.620	2.00000	2.29000	2.51500			
	Death (3)	2.480	2.950	2.49500	2.71000	2.93250			
Mitotic activity index	disease free (1)	2	8	2.00	3.50	5.50	11.678	**.001**	1,2 and 1,3
	Recurrence(2)	6	25	8.00	10.00	17.50			
	Death (3)	7	28	7.25	9.50	23.75			
M/V	disease free (1)	0.24	0.98	0.28	0.43	0.79	14.786	**<0.001**	1,2 and 1,3
	Recurrence(2)	1.07	3.85	1.20	1.51	3.23			
	Death (3)	2.14	4.52	2.25	3.03	4.25			

Table 2: Spearman's correlation between the five prognostic parameters.

Spearman's rho		M/V	IFG	Mean AgNORs number	Mean AgNORs area
Mitotic activity index	Correlation Coefficient	.885**	.728**	.526*	.611**
M/V	Correlation Coefficient	1.000	.769**	.630**	.639**
IFG	Correlation Coefficient		1.000	.757**	.636**
Mean AgNORs number	Correlation Coefficient			1.000	.634**

*-significance at the level of 0.05

** - significance at the level of 0.001

Table 3: Area under the Receiver operating characteristic Curve

Test Result Variable(s)	RECURRENCE	DEATH
IFG	**.833**	.750
Mean AgNORs number	.677	.828
Mean AgNORs area	.620	.891
Mitotic activity index	.813	.719
M/V	.729	.906
Combined Predictor	.656	**.953**

Legends

Table 1: Comparison of histological, mitotic and NOR variables with Prognosis using Kruskal Wallis test

Table 2: Spearman's correlation between the five prognostic parameters.

Table 3: Area Under the Receiver operating characteristic Curve.

Figure 1: 1000 point grid overlaid on the invasive front for assessment mitotic activity per volume of tumour.

Figure 2: AgNOR as seen in the histological section.

Figure 3: Box plot depicting the variation of the parameters between the disease free, recurrence and fatal groups of OSCC.

Figure 4: Scatter plot between the five prognostic parameters with each other.

Figure 5: ROC curves of the five individual parameter and combined formula derived.

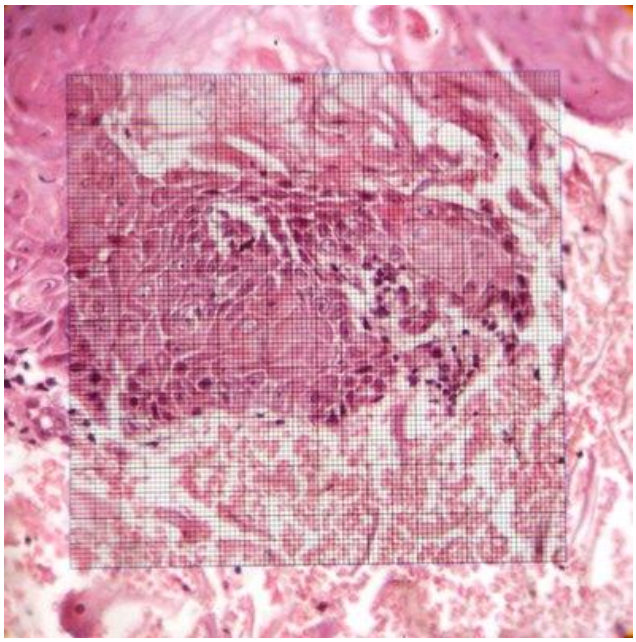

Figure 1

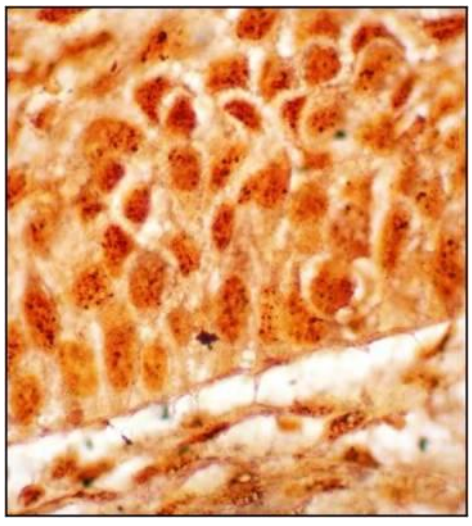

Figure 2

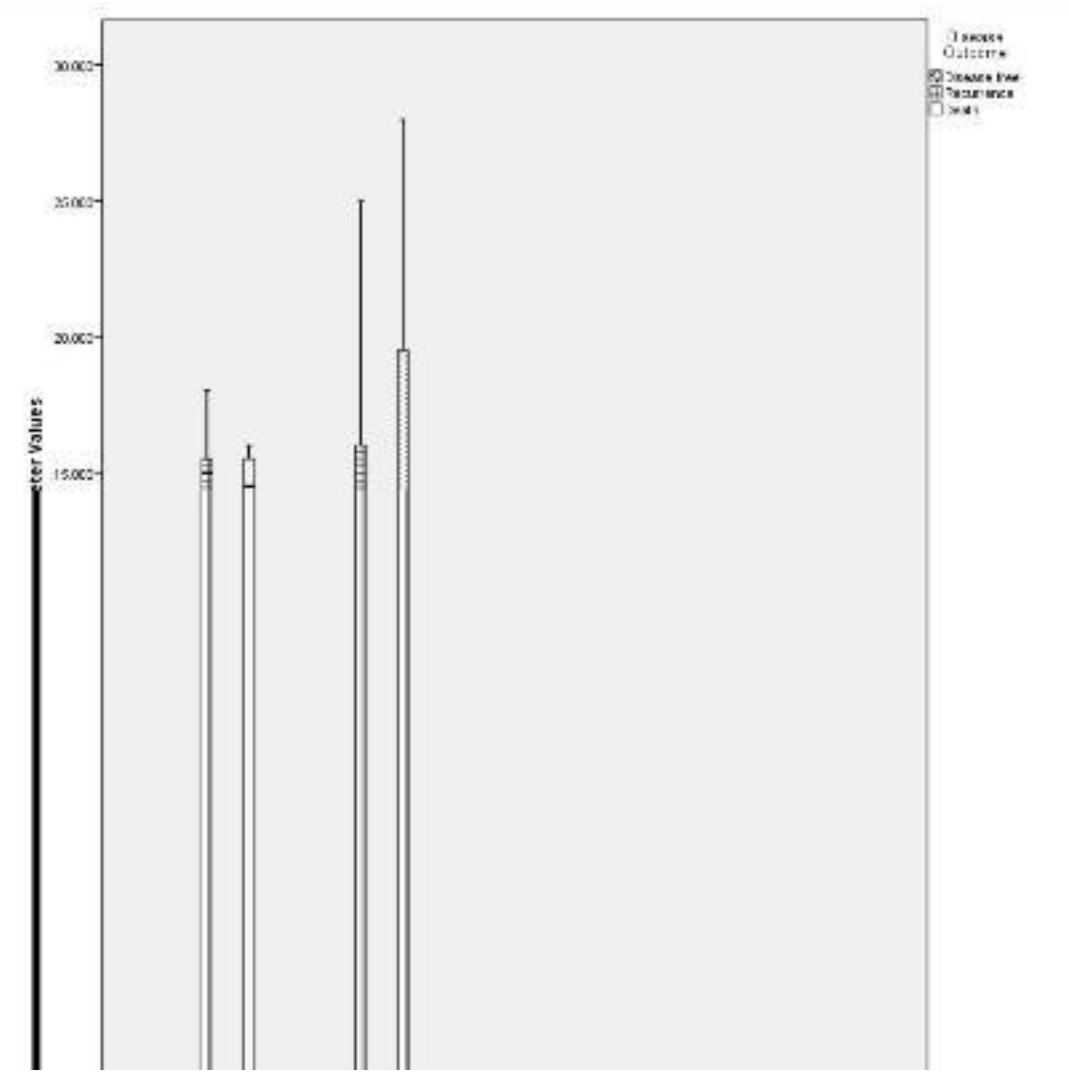

Figure 3

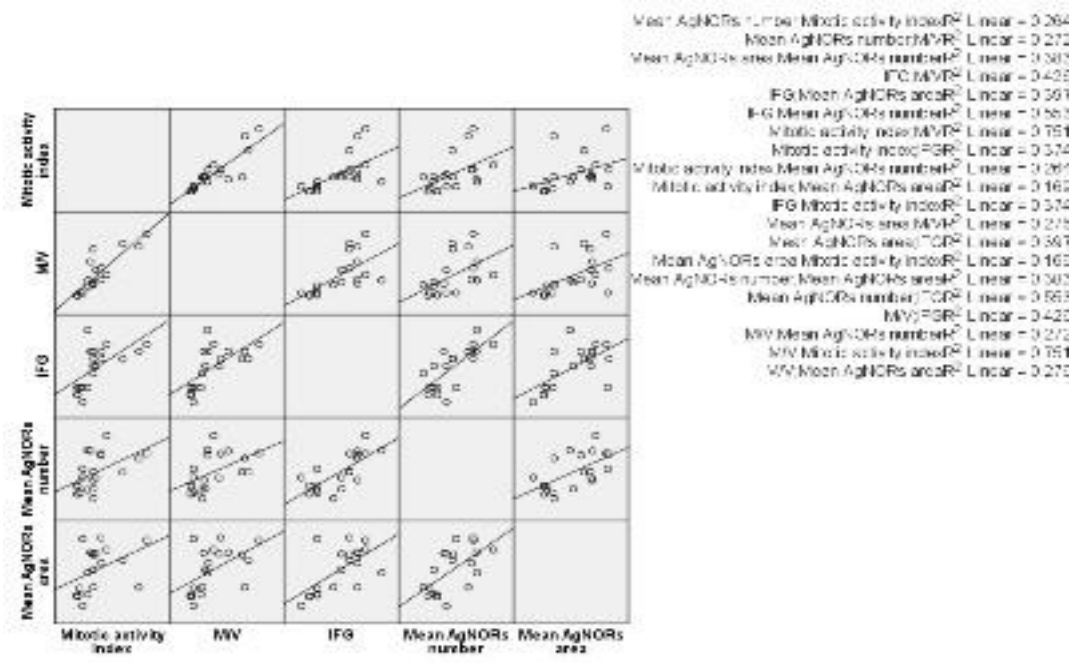

Figure 4

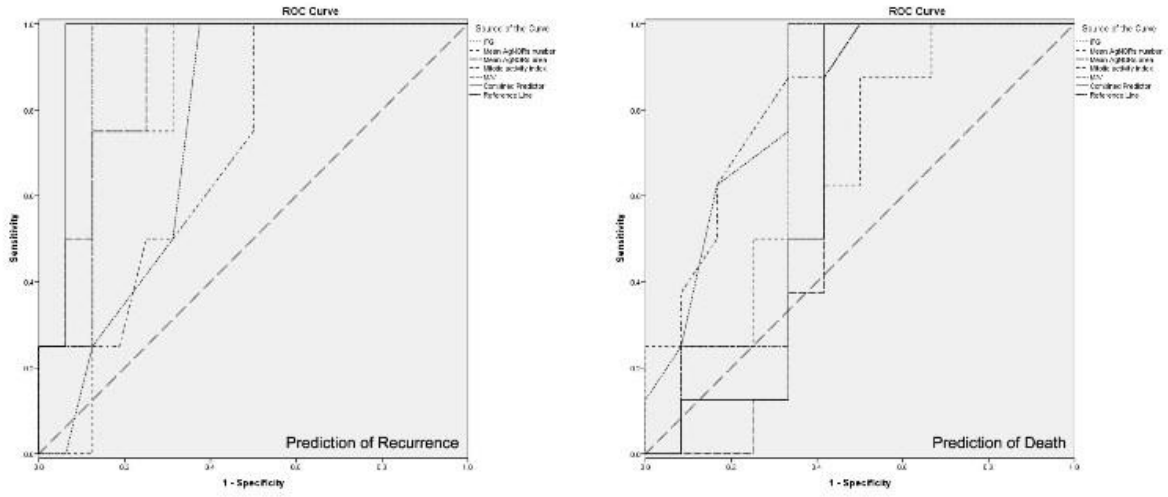

Figure 5