

Original Research Article

Title Page

Type of the manuscript: Original article

Title: A better perspective of nuclear atypia in Nottingham prognostic index: study on breast carcinoma patient cohort from Eastern India

Running title: Nuclear atypia in Nottingham prognostic index: breast carcinoma

Abstract

BACKGROUND: The widely accepted prognostic index for breast cancer, NPI has witnessed latest revisions to update the existing clinical prognostic index with that of molecular markers. However, very little is known about the independent prognostic power of the intermediate factors contributing to Nottingham prognostic index and the histologic tumor grade. **AIMS:** This paper uses the parameter-based correlation to NPI with specific attempt for histologic grade/ nuclear atypia. **MATERIAL AND METHODS:** In this paper we have done retrospective analysis of the histopathological data of 1372 breast carcinoma patients from eastern India and subjected to different regimens of therapy and patient health constitution driven treatment outcome. This paper uses the parameter-based correlation to NPI with specific attempt for histologic grade/ nuclear atypia. **STATISTICAL METHOD:** SPSS version 22. **RESULT AND CONCLUSION:** Nuclear atypia as seen from multivariate analysis and correlation study can be a better prognostic marker for a range of values with most the confidence interval being upto 3.71.

Keywords: Nuclear atypia; Histologic Grade; Nottingham Prognostic Index; Breast cancer; Tumor Size; Nodal Involvement.

Introduction

Clinical management of breast cancer depends on various clinical and pathologic prognostic and predictive factors to support clinical and patient decision making for suitable treatment options. It has been largely accepted by the clinicians that there is no exact generalization of an effective breast cancer treatment. The common modalities of treatment involve surgery, radiotherapy, chemotherapy, hormone therapy and biological treatment [1]. A modern and effective approach has been adopting a combinatorial approach for treatment of breast cancer based on the clinicopathological parameters involved and patient history along with bimolecular signatures associated with the disease manifestation [2]. Therefore, it is imperative to identify and characterize the commonly associated parameters of breast cancer like type of breast cancer; size of tumor; stage of tumor; grade of tumor; whether the patient had menopause, general patient health constitution and the status of cancer cell sensitive to receptor targeted therapy (trastuzumab) [3,4]. Although these parameters are mostly identified but the characterization along with correlation of these parameters with the most widely adopted prognostic index has

47 been negligible in the scientific community leading to a broad prognostic score
48 generation with limited applicability to treatment or early treatment options [5].
49

50 The Nottingham Prognostic Index (NPI), a continuous variable based index uses
51 retrospective factors like tumor size [6], nodal involvement [7] and tumor histologic
52 grade for determining the likelihood outcome of breast cancer patients [8].NPI has
53 been recognized as the only appropriately validated prognostic index in breast
54 cancer [9].The brief review of NPI gets clearer with the correlation summary of NPI
55 scores and the predicated survival rates (Table 1).

NPI Score	Survival rate (in %)
≥ 2.0 to ≤ 2.4	93
≥ 2.4 to ≤ 3.4	85
> 3.4 to ≤ 5.4	70
> 5.4	50

56 Table 1: Correlation between NPI Scores and Survival rate predicted through
57 Nottingham Prognostic Index. Higher the NPI score lower is the survival rate and
58 higher is the aggressiveness of breast cancer.
59

60 The prognostic index has not only facilitated stratification of treatment strategies
61 but also has been linked with the typical and significant molecular changes involved
62 in breast cancer. These molecular markers with stage specific expression patterns
63 have been developed using immunohistochemistry method for different clinical
64 samples of different stages of breast cancer (pre and post-surgery conditions) [10].
65 This improvised version of correlating the clinicopathological factors with that of the
66 molecular markers involved in progression of breast cancer has led to the
67 development of NPI-Plus (NPI+) [11,12]. In addition to predicting the treatment
68 outcome and deciding for next course of treatment it also aids in sensitive modeling
69 of treatment stage, patient health constitution and aggressiveness of cancer [13].
70 This bioinformatics modeling along with biological and clinical analysis has been the
71 basis of improvised prognostic index development for breast cancer [14].
72

73 Understanding the clinic-pathological factors involved in breast cancer has aided the
74 contemporary clinical management practices. One of the major outcomes of has
75 been categorical identification of individual factors and their summative and
76 independent correlation to prognostic index [15,16]. The three strongest prognostic
77 determinants in operable breast cancer used in routine clinical practice
78 internationally are lymph node (LN) stage, primary tumor size, and tumor histologic
79 grade, which constitute Nottingham prognostic index [17,18]. In 1957, Bloom and
80 Richardson proposed a simplified system, which utilized only three of Greenough's
81 variables: gland-formation (tubularity) degree of variation in nuclear size and shape
82 (pleomorphism) [19], and 'hyperchromatic figures' as an estimate of proliferation to
83 form the histological grade [20]. Though lymph node, which has traditionally been

regarded as the most powerful prognostic factor in breast cancer, all the other factors are potentially regarded as prognostic factor.

The histologic grade of tumor is primarily determined by nuclear atypia (nuclear pleomorphism), nodal involvement and mitotic activity [21]. Broadly, the histologic grade for breast cancer range from: Grade 1 (Low grade)-> Grade 2 (Intermediate grade)-. Grade 3 (High grade) [22]. Cancers with lower grade profile tend to grow slowly and respond much efficiently to surgery and other treatment regimens (radiotherapy, chemotherapy, hormone therapy, biological treatments) [23]. This ensures higher survivalrate, which thus can be deduced to be inversely related to NPI.

It is understood that the factors contributing to histologic grade have a correlation to the NPI. However, there has been no direct evidence of correlation of each of these factors or as independent parameters to NPI [24]. The Nottingham prognostic index, which has histologic grade as one of the constituting factors can be reasoned to have some significant and more indicative correlation to either of the factors making of histologic grade or a combination of those [25]. Nuclear atypia, nodal involvement and mitotic activity are three parameters making up the histologic grade [26,27] and more or less subjected to individual or contextual bias during observation or scoring [28,29]. However, the gap in this knowledge has limited our understanding and earlier prediction of treatment outcome or more clearly poorer prognosis.

This paper tries to identify the correlation of the factors making up histologic grade of tumors to that of the calculated Nottingham prognostic score [30,31]. The long-term aim of the work is to establish a better perspective to these factors other than mere calculation of histologic grade [32] and thereby develop any such significant factor for early prognosis in cases of breast carcinomas.

Methods

Patient Selection:

The samples were collected from 1372 primary invasive breast cancer patients for a period of 6 years starting from 2008 to 2013 from AcharyaHarihar Regional Cancer Research Centre and Hospital, Cuttack, Odisha. A series of histopathological analysis and data collection was subsequently followed up. The histopathological data was subjected to univariate, multivariate and parametric statistical analysis using IBM SPSS software version 22 (SPSS Inc., Chicago, IL, USA) [33,34].

The age of patients considered for the study ranged from 20 to 82 (Fig.1) years old of whom most were subjected to surgery/radiotherapy/chemotherapy and/or a suitable combination of these treatment options. The detailed patient characteristics and the parameter range used for this study has been summarized in Table 2. The sample processing involved initial clinical review of formalin fixed histological sections embedded with paraffin. This study was conducted under the regulations of Indian council of medical research. The handling of biological specimens from tumor

banks, being the samples available for research and clinical purposes in retrospective studies.

The histological diagnoses were cross-verified by trained pathologists. The tumor samples were categorical characterized for parameters like tumor size, nodal status, histologic grade, surgical margin, nuclear atypia, mitotic activity, (Table 2). In samples with valid data for all the parameters, NPI was calculated using the standard equation:

$$\text{NPI} = 0.2 \times \text{tumor size (cm)} + \text{grade (1-3)} + \text{lymph node status (1-3)}$$

In addition, patient history and medical related information were collected like age, sex, spread of breast cancer and their geographical origin (broadly defined in this paper as belonging to eastern India). Tumor size, histologic grade and the corresponding NPI are most frequent for their respective median values. The most frequent tumor size was 2-5 cm (86.7%) with the mean being 3.83 cm $\pm$ 2.00 cm (Fig.1). Similarly, histologic grade of tumors was mostly reported for grade 2 tumors (60%) indicating moderately severe aggressiveness of tumors among the cohort of patients.

The calculated NPI was most frequent for the range “3.4 $\leq$ NPI $\leq$ 5.4 and NPI $>$ 5.4” (Fig.1) indicating intermediate to poor survivability rates post treatment of the patients under consideration. With reference to the standard correlation of NPI with survivability rates (Table 1) it is assumed to be between 50-70% for the patient cohort under focus.

Table 2

Patient characteristics and tumor parameters

Variable (N = 1372)	Data
Age at Diagnosis, years	
Mean and standard deviation	47.06 $\pm$ 10.714
Range	62 (Min 20; Max 82)
Tumor Size (cm)	
Mean and standard deviation	3.83 cm $\pm$ 2.00 cm
Range	16.2 (Min 0.8; max 17)
T1: < 2 cm	94 (7.67%)
T2: 2-5 cm	1092 (86.7%)
T3: > 5 cm	469 (38.3%)

Not assessed	148
Lymph Node Invasion	
Present	657 (48.59%)
Absent	695 (51.40%)
Not assessed	20
Histological Grade	
Grade I	33 (2.50%)
Grade II	767 (60.10%)
Grade III	471 (37.0%)
Not assessed	101
Nottingham Prognostic Index	
NPI < 3.4	50 (4.14%)
$3.4 \leq \text{NPI} \leq 5.4$	566 (46.90 %)
NPI > 5.4	589 (48.80%)

156

157 **Statistical analysis:**

158 The histological data collected from the 1372 breast cancer patients was subjected
 159 to statistical analysis using IBM SPSS software version 22.0 (SPSS Inc., Chicago, IL,
 160 USA). The convention used in the data representation (Table 2) is: continuous
 161 variables have been represented in form of mean standard deviation and category-
 162 based variables have been represented as percentages.

163 The data was subjected to distribution analysis to ensure comparison uniformity
 164 among different parameters being used in this study, namely NPI; histologic grade;
 165 nuclear atypia; mitotic activity; nodal involvement and tumor size. An unpaired T-
 166 test with 95% confidence interval was used for generating the mean differences for
 167 continuous variables. The above specified parameters were subjected to univariate,
 168 multivariate and parameter bias analysis using SPSS. In addition to develop a
 169 predictive model for determining the range of NPI that can be predicted with the
 170 constituent factors making up histologic grade we performed a predictive analysis
 171 where the scores with 95% confidence was considered significant for our study. In
 172 this paper we have used Pearson correlation and chi-squared tests to determine the
 173 associativity of the parameters with NPI. (fig1)

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175

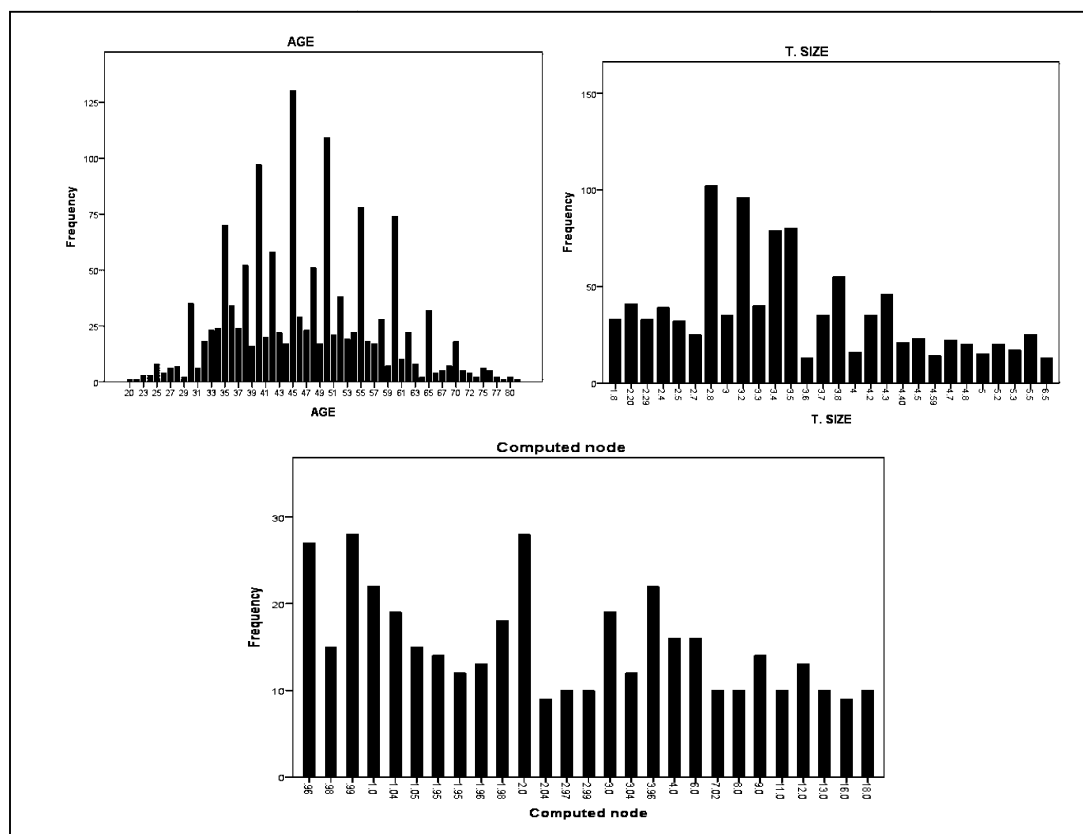


Fig.1. Distribution of the patient characteristics involved in the study of correlation of histologic grade parameters with NPI in 1372 breast cancer patients.

Result

1. Correlational Analysis: The study focuses on understanding the correlation of the Nottingham prognostic index (NPI) along with that of histologic grade as well as the factors contributing to it. However, the approach to deduce the correlation of the factors making up histologic grade independently to that of NPI has been an unique and oversearching attempt of this paper to understand for any clinical/ biological parameter that holds significant correlation to NPI and thus can be developed as an aid for early prognosis of breast cancer prior to standardization of NPI scores. The parameters giving rise to NPI like tumor size, lymph node involvement and histologic grade have also been attempted for finding any intuitive correlation. For the purpose of higher accuracy we have adopted the significance threshold to be at 0.01 level (2-tailed).

Of the parameters making up histologic grade only nuclear atypia and mitotic activity hold significantly high correlation (0.079 and 0.076 respectively) with that of NPI in contrast to tubule formation (Table-3). Thus, we build on our hypothesis that among three factors contributing to histologic grade, mitotic activity and nuclear atypia hold

stronger correlation and thus can be potential candidate for independent early prediction of treatment outcome in breast cancer patients. This correlational analysis or associativity study helps to build a more objective hypothesis that nuclear atypia or mitotic activity can be a better prognostic marker in contrast to tubule formation. In addition, the positively significant correlation also establishes a retrospective link of “not just histologic grade but factors making histologic grade can be better prognostic markers”. This adds higher prognostic power to the less explored parameters like nuclear atypia and mitotic activity.

Correlations

	PROGNOSTIC SCORE	T. SIZE	TUBULE FORMATION	NUCLEAR ATYPIA	MITOTIC ACTIVITY	Computed node	HISTOLOGIC GRADE
PROGNOSTIC SCORE	1	.022	.007	.076	.079	.034	.079
		.456	.819	.009	.006	.234	.006
	1202	1147	1184	1185	1184	1200	1185

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 3: Correlation Analysis of NPI with that of its constituent parameters (tumor size, nodal involvement and histologic grade).

2. Sample distribution study and range homogenization:

Considering the ample number of sample, i.e. 1372 samples from eastern Indian cohort of patients, it is imperative to understand that the difference in sample exposure to climate, genetic make up, epigenetic influencers as well as the experimental bias do not significantly mask the correlation outcome. So, it was hypothesized that identification of the frequency distribution patterns (Fig2) and the correlation of the various categories with that of histologic grade and the parameter of focus i.e. “nuclear atypia” will enable us to confirm the applicability of our data to clinical set up and to homogenize the differential missing data for each parameters among all the samples.

Frequencies w.r.t Histologic Grade (SBR)

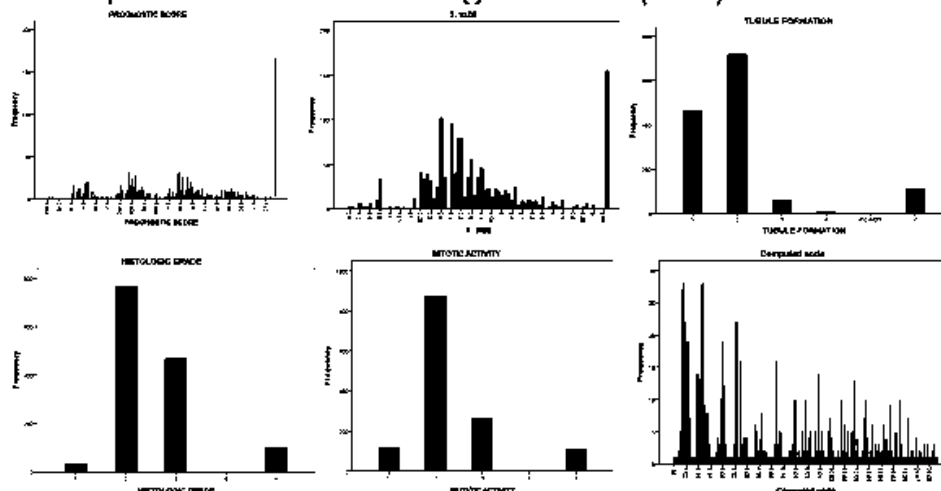


Fig.2. Frequency distribution of breast cancer parameters like NPI, Tumor size, tubule formation, mitotic activity and nodal involvement with that of histologic grade/nuclear atypia.

The NPI as well as the inherent factors like tumor size and nodal involvement have a similar pattern of distribution with the most of the samples being crowded around the median case for each. With respect to histologic grade there is a significant mismatch of the range of sample distribution of NPI and histologic grade where histologic grade is mostly accumulate within grade I and grade II tumors. So the applicability of NPI for prognosis forecasting might lead to erroneous assumption and clinical bias during treatment.

However, nuclear atypia has a much similar frequency distribution pattern in comparison to NPI as well as other parameters like tumor size, nodal involvement and tubule formation.

3.Histologic grade and the role of contributing parameters:

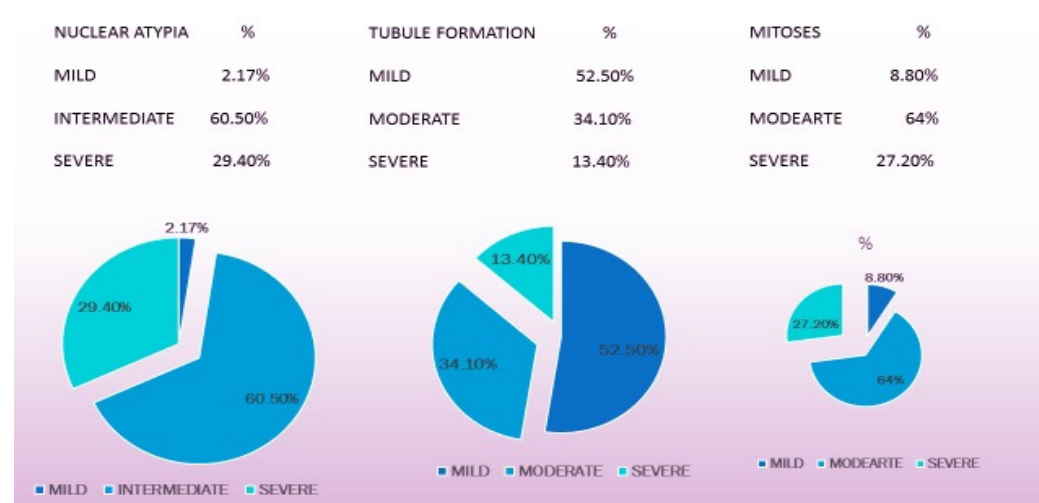


Fig.3. Frequency distribution of breast cancer parameters like NPI, Tumor size, tubule formation, mitotic activity and nodal involvement with that of histologic grade/nuclear atypia.

Histologic grade of tumors is the summation of three parameters observed in tumors like nuclear atypia, tubule formation and mitotic activity. As deciphered from the correlation analysis (Table 3) nuclear atypia and mitotic activity have higher significance and independent correlation to that of NPI and thus characterizing the pattern of this categorical variable for the continuous variable (NPI) is important to deduce a meaningful relationship.

Nuclear atypia and mitotic activity as like NPI also hold their most frequent observation in the median range, which means a case of intermediate nuclear pleomorphism or atypia and mitosis. This suggests that nuclear atypia or mitotic activity or both can have a stronger predictive correlation depending how significant is their univariate/ multivariate analysis.

4.Univariate Analysis:

Univariate analysis with NPI as the dependent variable subjected to parametric variations highlight histologic grade to be the better prognostic marker in comparison to other parameter like nuclear atypia, tubule formation and mitotic activity (Table 4). NPI has a high significant correlation with histologic grade 0.864 in comparison to nuclear atypia (0.421) and tubule formation (0.439). The result of univariate analysis supports the contemporary convention that the "histologic grade itself can be an early prognostic factor than that of inherent factors like nuclear atypia and mitotic activity". However, modeling the prognostic index using univariate analysis limits the variability of the multiple parameters and doesn't resemble or recreate the practical influence of multiple parameters on NPI. Therefore, we proceed with multivariate analysis.

Tests of Between-Subjects Effects

Dependent Variable: PROGNOSTIC SCORE

Source	Mean Square	Sig.
TUBULEFORMATION	108.141	.439
NUCLEARATYPIA	117.178	.421
MITOTICACTIVITY	310.858	.190
T.SIZE	32.491	.671
HISTOLOGICGRADE	5.312	.864
Computednode	242.327	.234

**. Correlation is significant at the 0.01 level (2-tailed).

Table 4: Univariate correlation analysis of NPI with that of its constituent parameters (tumor size, nodal involvement, histologic grade, nuclear atypia and mitotic activity).

5. Multivariate Analysis:

Parameter Estimates

Dependent Variable	Parameter	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
PROGNOSTIC SCORE	Intercept	2.760	.878	-5.839	4.991
	HISTOLOGICGRADE	1.398	.337	-1.401	4.085
	NUCLEARATYPIA	1.454	.555	-1.995	3.711
T. SIZE	Intercept	.319	.000	2.377	3.627
	HISTOLOGICGRADE	.161	.004	.145	.778
	NUCLEARATYPIA	.168	.617	-.413	.245
Computed node	Intercept	1.217	.005	1.034	5.808
	HISTOLOGICGRADE	.604	.248	-.487	1.882
	NUCLEARATYPIA	.634	.849	-1.365	1.123
MITOTIC ACTIVITY	Intercept	.083	.000	.844	1.171
	HISTOLOGICGRADE	.042	.000	.843	1.008
	NUCLEARATYPIA	.044	.000	-.407	-.235
TUBULE FORMATION	Intercept	.118	.000	1.822	2.286
	HISTOLOGICGRADE	.060	.000	-.494	-.259
	NUCLEARATYPIA	.062	.013	.033	.277

**. Correlation is significant at the 0.01 level (2-tailed).

Table 5: Multivariate analysis of NPI, tumor size, nodal involvement, mitotic activity and tubule formation with that of histologic grade and nuclear atypia.

A multivariate analysis of NPI with that of histologic grade and nuclear atypia reveals a contradictory or more suggestive explanation to the univariate analysis result (Table 5). Here nuclear atypia is more significantly related to NPI score than that of histologic grade but the confidence interval for histologic grade runs higher than that for nuclear atypia. Nuclear atypia for the sample of this study mostly range in the intermediate range (Fig.3) therefore a confidence threshold of 3.711 permits only type 1 and type 2 cases of nuclear atypia. However, histologic grade with confidence threshold of 4.095 accounts for most of the reported tumor grades, which range among grade 1/2/3 and most frequently in this study for grade 2. Thus, in a way histologic tumor grade has a wider window for accommodating higher number and variety of samples for better prognosis for most of the breast cancer patients whereas nuclear atypia, is limited by the specific confidence limit for upto 3.711.

6. Non-Parametric Analysis:

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of PROGNOSTIC SCORE, T. SIZE and HISTOLOGIC GRADE are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Table 6: Non-parametric analysis of NPI, histologic grade and nuclear atypia using Friedman's Two-way Analysis of variance by ranks.

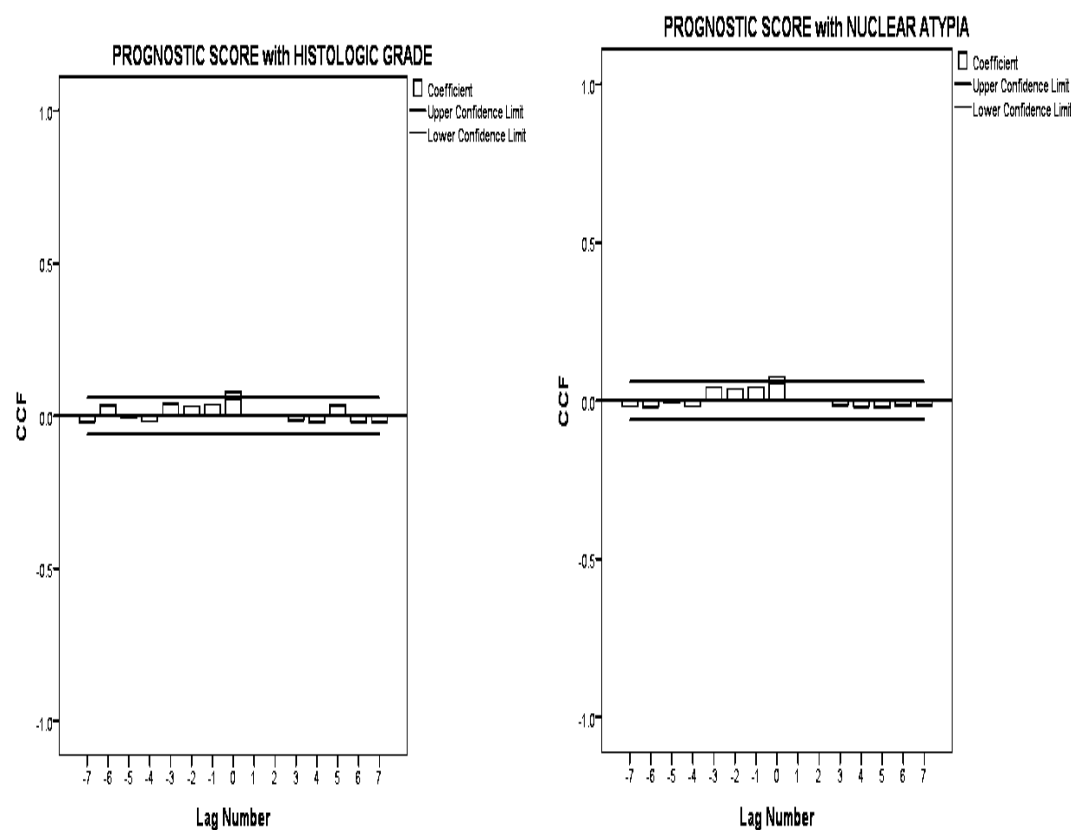
In order to account for and eliminate the bias of any parameter towards NPI and the model of multivariate analysis with NPI as the dependent variable and histologic grade and nuclear atypia as the parameters is subjected to Friedman's Two-way analysis of variance by ranks. The result indicates that no significant parametric bias exists in the multivariate analysis model used above (Table 5) and thus the null hypothesis, i.e. "NPI, Tumor Size and Histologic grade" are the same for nuclear atypia stands rejected.

7.Predictive analysis of NPI w.r.t to histologic grade/ nuclear atypia.

The correlational analysis and variable study of NPI with that off histologic grade and nuclear atypia suggests for possible methods for early prognosis of breast cancer from clinical data. Therefore, modeling in retrospective manner will help to generate a predictive model for estimating the treatment outcome of patient and thereby deciding how reliable can the nuclear atypia based prognosis can be in face of the 95% confidence interval being exerted upon.

The confidence co-efficient (CCF) of NPI with respect to histologic tumor grade and nuclear atypia help in a developing a reference based model for predicting the treatment outcome. The model as shown in Fig.4 represents the table of NPI correlation to histologic grade or nuclear atypia and the reliability of the prediction using 95% confidence interval as the filter.

324



325 Fig.4. Predictive analysis model of NPI using histologic grade/ nuclear atypia as
 326 significant parameter estimates.

327

328 Discussion

329

330 The Nottingham prognostic index has been the most widely accepted and used
 331 prognosis index for primary operable breast cancers around the world. Several
 332 experimental data arising from different geographical coordinates of the world have
 333 confirmed the validity of NPI and simultaneously few of these have added
 334 exceptions or newer possibilities to the NPI. In this paper, we tried to understand
 335 how histologic grade (considered to be an independent prognostic parameter in
 336 comparison to tumor size and node status) correlates to NPI. Precisely, we have
 337 explored the patterns of possible correlation of parameters making up histologic
 338 tumor grade and NPI.

339

340 From the correlational analysis the results gave a clear indication that indeed the
 341 correlation of NPI and histologic grade bears statistical significance and especially
 342 nuclear atypia and mitotic activity (factors constituting histologic grade) are two of
 343 those parameters with higher significance of correlation with NPI. Further since the
 344 data set for there study consisted to heterogeneous valid data for each parameters,
 345 it was necessary to compare the distribution and frequency of the parameters

including NPI to eliminate experimental error and input / output mismatch. The data of 1372 breast cancer patients suggested that most of the data remained in the median distribution range and the nature of tumor under study ranged from intermediate to severely aggressive ones with lesser survivability rates and poorer treatment outcomes.

Associativity analysis using univariate and multivariate analysis models suggest that nuclear atypia than the overall histologic grade holds significance in early and better prognosis of breast cancer patients but the confidence levels with which NPI can be predicted using either of these as parameters varies. Histologic grade has a wide range of grade values for which NPI can be predicted with more than 95% confidence score whereas nuclear atypia experiences a lower threshold cut-off at just 3.711 for predicting NPI. This suggests practical limitation of nuclear atypia in predicting prognosis for intermediate and severely aggressive breast cancers where the survival rates are low. In other words, nuclear atypia as an independent prognostic marker serves good when used for predicting grade 1/ grade 2 tumors with NPI ranging 5.4 or less. Since, most of the cases of less aggressive breast cancers are hard to detect and less reported the clinical appreciation of nuclear atypia, as an important prognostic factor has been less. In addition the non-parametric analysis suggested there is no statistical parametric based bias as nuclear atypia recognizes NPI, tumor size and histologic grade to be independent parameters and correlates differentially with each of these.

Future Perspective

It would be expected in the scientific and clinical domain that any prognostic tool developed must be applicable to all the known forms of breast carcinomas. However, one of the major limitations to developing NPI is the inability or the paradoxical applicability to triple negative breast cancer patients (insensitive to estrogen, progesterone and trastuzumab targeted therapy).

The existing results of triple negative breast cancer suggest that there is a prevalence of lymph node metastasis at the time of diagnosis. In contradiction few other literatures suggest that the metastasis and spread of cancer cells in triple negative breast cancer have a heterogeneous preference and doesn't always spread to lymph nodes. Breast cancer in general is characterized by heterogeneity of patient outcome in terms of response to treatment and their health constitution. However, this heterogeneity is further complicated in triple negative breast cancer patients.

Conclusion

A future perspective of developing NPI is to identify and characterize the prognostic patterns associated with triple negative breast cancers and specifically characterize for cases with lymph node metastasis and those without. This retrospective analysis will allow for a more widely accepted form of NPI with better applicability to triple negative breast cancer patients.

393 **Ethical approval**

394 Not applicable

395

396 **Conflict of interests**

397 Authors declared that no conflict of interests exists.

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399 **Consent**

400 Not applicable

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