Impact of intratumoural heterogeneity on the assessment of Ki67 expression in breast cancer

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Abstract

In breast cancer (BC), the prognostic value of Ki67 expression is well-documented. Intratumoural heterogeneity (ITH) of Ki67 expression is amongst the several technical issues behind the lag of its inclusion into BC prognostic work-up. The immunohistochemical (IHC) expression of anti-Ki67 antibody (MIB1 clone) was assessed in four full-face (FF) sections from different primary tumour quadrants and their matched axillary nodal (LN) metastases in a series of 55 BC. Assessment was made using the highest expression (hot) spots (HS), lowest expression (LS), and overall/average expression scores (AS) in each section. Heterogeneity score (Hes), co-efficient of variation and correlation coefficient were used to assess the levels of Ki67 ITH. Ki67 HS, LS, and AS scores were highly variable within the same section and between different sections of the primary tumour, with maximal variation observed in the LS ($p<0.001$). The least variability between the different slides was observed with HS scoring. Although the associations between Ki67 and clinicopathological and molecular variables were similar when using HS or AS, the best correlation between AS and HS was observed in tumours with high Ki67 expression only. Ki67 expression in LN deposits was less heterogeneous than in the primary tumours and was perfectly correlated with the HS Ki67 expression in the primary tumour sections ($r=0.98$, $p<0.001$). In summary, assessment of Ki67 expression using HS scoring method on a full-face BC tissue section can represent the primary tumour growth fraction that likely to metastasise. The association between Ki67 expression pattern in the LN metastasis and the HS in the primary tumour may reflect the temporal heterogeneity through clonal expansion.

Introduction

Immunohistochemical (IHC) detection of proliferation associated antigens have long been studied in breast as well as other cancers [1]. Ki67 is a labile non-histone nuclear protein of approximately 395 kDa, amount of which is tightly regulated during cell cycle. [2-4] Detailed cell cycle analysis revealed its presence in the nuclei of cells in all active cycling/proliferative phases, while quiescent/resting cells do not express it. Therefore, it has been used as a biomarker to assay the growth fraction of a given cell population [1, 5]. Nuclear positivity of Ki67 protein is an indication of cell proliferation[6, 7] and it has long been reported as a prognostic marker in breast cancer (BC) [8-11]. There is evidence that tumours with higher expression levels of Ki67 respond better to adjuvant chemotherapy than those tumour having low levels of Ki67 [11, 12]. Ki67 expression is an independent predictive of neoadjuvant chemotherapy in BC patients [13, 14]. as well as the neoadjuvant endocrine therapy in postmenopausal patients.[15] Patients with high post-treatment Ki67 expression levels have a higher risk for disease relapse and death than those with low or intermediate Ki67 expression [16].

Ki67 is used to provide additional useful prognostic information in BC: 1) to define the intrinsic molecular subtypes immunohistochemically [17, 18], 2) as a component in the prognostic multigene signatures including Oncotype DX [19] and IHC4 [20, 21], and 3) prognostically stratify grade 2 tumours [22] and accurately reflect tumour proliferative status in poorly fixed specimens. However, biological heterogeneity of Ki67 expression is a well-documented phenomenon visible across specimens from the same tumour. Such heterogeneity could be a gradient of increasing Ki67 positivity toward the tumour edge and or Ki67 variable expressivity anywhere in the tumour in the form of hot spots [23, 24]. To ensure satisfactory reproducibility when using Ki67 IHC in research

purposes and in the way of it inclusion into routine clinical practice for BC, it is still debatable whether to score the hot spots of expression or the average expression. The International Ki67 in Breast Cancer working group recommends scoring Ki67 as the average score of positive nuclei in the invasive tumour. This was based on the lack of sufficient evidence to use the "hot spot" of expression [24]. Moreover, many of the previous Ki67 prognostic studies have used tissue microarray [25, 26], which may not represent the proliferative status of the whole at individual patients level. In this study, Ki67 expression was assessed on full-face sections from different primary tumour quadrants of surgical specimens as well as from the corresponding axillary nodal metastasis. A systematic approach of staining assessment was followed to assess the impact of intratumoural heterogeneity of Ki67 expression in the same section as well as different sections from multiple primary tumour blocks and corresponding nodal metastatic deposits.

Materials and methods:

In this study, four different primary tumour quadrants from 55 primary operable invasive breast carcinomas and sections from the corresponding axillary nodal metastases were used to assess Ki67 expression. This subset of cases is from the Nottingham primary series of early invasive BC series from patients presented to Nottingham City Hospital from 1995-1998 and managed according to a uniform protocol. Cases were chosen based on the availability of four tumour blocks per tumour. Tumours in these patients were 5 cm in diameter or less at time of presentation [27]. Patients' clinical and pathological data including age, histological tumour type, primary tumour size, lymph node status, histological status, Nottingham prognostic index (NPI), and vascular invasion were available and prospectively maintained [28-30]. This study was approved by Nottingham Research Ethics Committee 2 under the title of ''Development of a molecular genetic classification of breast cancer''.

Immunohistochemistry:

Form each Formalin-fixed paraffin embedded tissue block, 4 μm thick full-face tissue sections were cut and mounted on Superfrost slides (Surigpath). The immunohistochemical (IHC) expression of Ki67 was determined using MIB1 antibody (MIB1 clone, M7240, DAKO, Denmark) and the Novolink™ Max Polymer Detection System from Leica Biosystems (Leica Biosystems, RE7150-K, Leica, Newcastle, UK). Heat induced retrieval of antigen epitopes was performed in citrate buffer (pH 6) using microwave for 20 minutes, followed by immediate cooling. MIB1 primary antibody diluted as 1:100 in Leica antibody diluent (RE7133) was incubated for 60 min at room temperature. As per manufacturer's guidelines, 3-3' Diam-inobenzidine tetrahydrochloride (Novolink DAB substrate buffer plus) was freshly prepared and used as a chromogen. Sections were counterstained with Mayer's haematoxylin for 6 min.

Negative (primary antibody replaced by phosphate-buffered saline) and positive controls (human tonsil sections) were included in each staining run to ensure reproducibility. Slides were dehydrated in alcohol, cleared in xylene and mounted with DPX.

Ki67 scoring:

Ki67 stained FF sections from different tumour quadrants and the matched axillary metastasis in lymph node (LN) positive cases were assessed following the Highest Score (HS = Hot-Spot), Lowest Score (LS), and Average Score/overall (AS) approach. Each section was first scanned at low-power light microscopic examination to identify area of HS and LS. As per our previous report, the HS was assessed in 1000 malignant invasive BC cells within the areas of hot spot staining at high power magnification (400×) [12]. The LS reflected the Ki67 score in the area of least number of positively stained malignant nuclei. The AS was the overall Ki67 scoring within the whole FF section taking into account all areas of tumour positive expression. The HS, AS, and LS were expressed as percent of Ki67-positive tumour cells divided by the total number of tumour cells within the assessed tumour area of invasive tumour. For each section, heterogeneity Score (HeS) was calculated as the difference between HS and LS (HeS = HS-LS). In all these scoring approaches, only nuclear staining in invasive tumours cells irrespective of its intensity was considered positive.

Statistical analysis

The statistical analysis was performed using Statistical Package for Social Sciences SPSS version 21 for Windows (Chicago, IL, USA). Co-efficient of variation (cvar), HeS, and Spearman rank correlation co-efficient were used to assess the ITH of Ki67 expression within the same section, between different quadrants from the same case, and between

the primary tumour and LN metastases. A p value of less than 0.05 (two-tailed) was considered significant.

Results:

In the current study, the median patients' age at diagnosis of this cohort was 52 years (range 30–70 years), with up to 53% of patients postmenopausal. The vast majority of cases were grade 2 and 3 (95%). Of all tumours, 35/55 cases (63.6%) were ductal carcinoma no special type (ductal/NST), with the remainder consisting of invasive lobular and mixed carcinomas. ER positive tumours constituted (69.1%), while 13 cases (24.1%) were HER2 positive (based on IHC and HER2 gene amplification detection using CISH). Supplementary Table 1 summarises patient demographics and clinicopathological criteria of their tumours.

Within the studied sections, Ki67 showed highly variable expression. Figure 1 depicts the LS and HS in different microscopic fields of two different BC cases. The Ki67 scores within the same section and in sections from different tumour quadrants in this series ranged from 0 to 100%. However, Ki67 staining within the metastatic deposits was more or less homogenously distributed and the AS ranged from 10-95%.

To assess the level of heterogeneity, HeS was calculated for each section from each quadrant. Table 1 shows the ranges, mean, and median of HeS for each of the sections from different tumour quadrants of the studied series. The median HeS (i.e. the difference between the HS and LS in each section) for each quadrant was: 1, 2, 3 and 4 was 25, 20, 20, and 25, respectively. The intra-slide HeS scores were significantly higher in cases with NST histology than invasive lobular and mixed cases (ANOVA, F = $P<0.001$) denoting higher Ki67 HS expression. However, the inter-slide HeS scores were significantly less variable in NST than the other two histologic variants, Figure 2.

The co-efficient of variation (CVAR) between Ki67 HS, LS, and AS within the same section (intra-slide scores) ranged from 13-96%. These figures demonstrated wide range of spatial heterogeneity of Ki67 expression within the same section of each tumour quadrant. To study the level of variability between HS, AS, and LS in different quadrants (inter-slide scores), the CVAR showed the least variability between the HS in different quadrants while the AS and LS showed higher variability, Figure 3. Levene's F test for homogeneity of variance showed statistically significant differences between CVAR of HS, AS, and LS in the four studied quadrants from each case (F= 6.639, P = 0.002). Tukey post-hoc analysis test showed that the CVAR of the HS were not statistically significant from those of the AS for the four quadrants studied (P = 0.835), while CVAR of the LS were significantly different from those of HS (P = 0.002) and AS (P = 0.014), Table 2.

We proceeded to study the differences between Ki67 AS and HS and tested their statistical associations as continuous variables with the standard prognostic parameters. Statistically significant associations were observed between the HS and tumour grade, mitotic scores, nuclear pleomorphism, histologic tumour type, ER status, PR Status, HER2 status, and BC molecular subtype as previously defined [28], more than with the Ki67 AS. However, no significant associations were observed between Ki67 AS or HS and menopausal status, tumour size, axillary nodal stage, NPI classes, or lymphovascular invasion (LVI; Table 3)

Because Ki67 is often dichotomised into a categorical variable, Ki67 AS and HS scores were categorised into low and high at 14% cut-off point. At this cut-off, cases tended to be classified as low proliferative with the AS relative to the HS within the same section. Table 3 displays the Ki67 AS and HS as classified into low and high at 14%, Table 4.

Ki67 staining within the metastatic deposits in axillary nodes was more or less homogenously distributed and the Ki67 expression ranged from 10-95%. Spearman rank correlation co-efficient of Ki67 expression of metastatic deposits in the studied LN sections showed significant positive correlation with Ki67 expression in the studied primary tumour sections. Although the AS was significantly correlated with Ki67, the strongest correlation ($r=0.98$, $p<0.001$) was observed with the HS of the primary tumour, Figures 4.

Univariate survival analysis of categorised Ki67 scores showed similar associations between Ki67 HS at 10% and 14% cut-offs and outcome in the different FF sections of the primary tumours and this was comparable to the associations obtained with mitotic scores.

Discussion:

The proliferative marker Ki67 is a molecular maker with documented prognostic utilities in BC singly and in combinations with other markers [31]. Because only cycling cells express it [32], its heterogeneous distribution in invasive BC is anticipated. Highly proliferating tumours are likely to behave more aggressively but respond better to cytotoxic chemotherapy compared to low proliferating tumours [33]. However, the behaviour and response to antimitotic drugs of high and low proliferating clones within the same tumour remain unclear. In BC it is common to see higher proportions of Ki67 positive cells at the periphery of the tumour denoting higher proliferation rates with areas more proliferative than others leading to the appearance of HS. Tumour areas in between HS show lower proliferative activity whilst lowest proliferative areas are called LS. Assessment of the overall Ki67 expression including HS and LS and areas in between is called average score. Currently no consensus for Ki67 staining evaluation whether it should only consider the HS if present, take the average score including HS or avoiding them completely [23] and comparable study assessing different scoring methods is needed.

In this study, we used the validated MIB1 as anti-Ki67 monoclonal antibody, recommended as being the gold standard for its enhanced specificity [24], to assess the pattern of expression of Ki67 in a subset of BC using full-face sections from different primary tumour quadrants. This was primarily to test for spatial intratumoural heterogeneity (ITH) and its impact Ki67 assessment. In addition, matching cases with positive nodal metastasis were assessed as an endpoint to assess metastatic tumour cell clones. In each of the stained sections, expression was assessed in the HS, LS, and the AS.

Within the studied cases, highly variable Ki67 expression was evident within the same section as well as in sections from multiple tumour quadrants representing spatial

zones in the primary tumour. Nevertheless, within the nodal metastatic deposits, staining showed homogenous distribution and was more towards the high proliferative side. Highly variable Ki67 expression was evident, both visually and statistically as per the co-efficient of variations results, within the same section; the intra-slide scores, as well as in sections from multiple tumour quadrants, the inter-slide scores, representing spatial zones in the primary tumour. Interestingly, inter-slide differences of AS, and LS were statistically significant in the four quadrants from each case, while the HS were not significantly different. Moreover, pairwise differences revealed that the LS were significantly different from those of HS and AS, while the latter were not different from each other. This latter finding underscores the impact of sampling on the final status of Ki67 being high or low. For instance, some studies reported on Ki67 prognostic significance were conducted on tissue microarrays (TMA) [34, 35]. TMA randomly punches out tiny cores, typically 0.6mm, which could hit low proliferative areas, yielding a low Ki67 status in an otherwise high proliferative case. Although TMAs are primarily a research tool conducted on large number of patients in population-based studies, this sampling error is usually balanced for by the large number of study population giving an overall prognostic significance [36, 37]. However, generalising cut-offs demarcating low from highly proliferative cases generated in studies using TMA carries the potential of misclassification of some patients from high to low proliferative subgroups [38]. However, at an individual patient level, which is the case in clinical decision making for neoadjuvant therapy using core needle biopsy (CNB), again sampling bias or error could influence the final status of the case, as shown in our study. Fair agreement for Ki67 assessment between CNB and surgical specimen has been reported [39], denoting that the former may not truly representing the biologic profile of the tumour. Accordingly, specimen specific cut-off point (s) is to be considered. For

instance, when using preoperative CNB or when translating data from TMA-based research studies to potential clinical uses.

To assess whether the Ki67 HS and AH could be interchangeably used, their associations, as continuous variables, with other BC prognostic and molecular parameters more significant associations were observed of the former than the latter. In this study, using different cut-offs, cases tended to be misclassified as low proliferative with the AS relative to the HS within the same section. Taken together, the HS was more representative of tumour proliferative status than the AS; results consistent with other reports using single FF section [40].

Within the nodal metastatic BC cells deposits, Ki67 staining showed homogenous distribution and was more towards the high proliferative side. Also, near perfect significant direct correlation was observed between Ki67 HS of the primary tumour and Ki67 score in metastatic nodal deposits. It was observed that the cells within the nodal deposits were highly proliferative and coincided with the HS of the primary tumour. As metastatic spread develops over time with natural selection of more aggressive clones [41], our findings could represent a further supporting evidence to endorse the Ki67 HS scoring approach as they are more representative of the metastatic clones.

We have previously reported on prognostic utility of Ki67 in a large series of BC [12, 42]. However, in this cohort, survival analyses showed shorter outcomes in patients with high Ki67 HS in the different sections from the primary tumour, yet the differences did not reach statistical significance. This could be attributed to the relatively limited numbers of patients when sub-grouped into low and highly proliferative. This is supported which is supported using mitotic scores of the same set yielded the same results.

Conclusions: In this study, the spatial heterogeneity of Ki67 expression in invasive BC was evident within at intra-slide as well as inter-slide levels using full-face sections from the primary tumours. The high Ki67 within LN metastasis corresponded to highest primary tumour expression reflecting tumour heterogeneity during metastatic process through clonal expansion. Therefore, the HS/hot spot scoring in full-face sections is more representative of the primary breast cancer growth fraction.

Conflicts of interest

The Authors have no conflicts of interest to declare.

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Figure legends:

Figure 1: IHC expression of Ki67; A and B: LS and HS in an invasive BC in different fields of the same section. C and D another example of LS and HS of the same BC in different fields of the same section**.**

Figure 2: median Heterogeneity scores (Hes) scores in multiple primary tumour quadrants in histologic tumour types (no special type NST, mixed types, and lobular carcinomas).

Figure 3: Box plots for coefficient of variation of Ki67 HS, AS, and LS scores in multiple tumour quadrants. HS: Hotspot score, AS: average score, LS: lowest score.

Figure 4: IHC expression of Ki67 in the primary tumour and LN metastatic deposits: A) LS, B) HS of the primary tumour, C) Ki67 expression in metastatic axillary nodal deposits, and D) Spearman rank correlation of Ki67 HS in the primary tumour and in the metastatic deposits in axillary LN.

Tables

Table 1: Ki67 Heterogeneity scores (Hes) for each of the full-face sections from different tumour quadrants of the studied series.

	Range	**Mean**	**Median**
Hes Quadrant 1	4 - 55	25.38	25
Hes Quadrant 2	1- 60	24.11	20
Hes Quadrant 3	3 - 70	25.49	20
Hes Quadrant 4	3 – 60	24.71	25

Table 2: Levene's F test for homogeneity of variance showing multiple and pairwise comparisons of coefficient of variation (CVAR) of HS, AS, and LS of Ki67 scores in the four quadrants of each of the studied case

SCORE	Pairwise Comparison	S Error	Significance, P
CVAR HS 4 Q	CVAR AS 4 Q	0.03711	0.835
	CVAR LS 4 Q	0.03711	0.002
CVAR AS 4 Q	CVAR LS 4 Q	0.03711	0.014

Table 3: Statistical associations between Ki67 HS and AS and the clinicopathological parameters of the studied series:

Parameter	Quadrant1 Significance P (test)		Quadrant2 Significance P (test)		Quadrant3 Significance P (test)		Quadrant4 Significance P (test)	
	HS	**AS**	**HS**	**AS**	**HS**	**AS**	**HS**	**AS**
Age*	0.641 (347.5)	0.845 (363.5)	0.520 (413.0)	0.492 (415.5)	0.926 (380.5)	0.721 (396.0)	0.461 (129.0)	0.731 (141.0)
Menopausal Status*	0.813 (363.0)	0.993 (377.5)	0.384 (428.5)	0.393 (427.5)	0.685 (401.0)	0.559 (411.5)	0.782 (144.0)	0.935 156.0)
Tumour Grade**	< 0.001 (25.5)	< 0.001 (23.4)	< 0.001 (25.5)	< 0.001 (24.3)	< 0.001 (27.5)	< 0.001 (27.5)	< 0.001 (21.8)	< 0.001 (25.3)
Pleomorphism*	< 0.001 (498.0)	< 0.001 504.0)	< 0.001 (480.0)	< 0.001 (476.5)	< 0.001 (492.5	< 0.001 (498.0)	< 0.001 (218.5)	< 0.001 (221.0)
Tubule Formation*	0.227 (377.0)	0.244 (374.5)	0.246 (374.5)	0.212 (379.0)	0.089 (403.5)	0.202 (380.5)	0.299 (161.5)	0.268 (163.5)
Mitotic Figures**	< 0.001 (36.5)	< 0.001 (34.4)	< 0.001 (37.7)	< 0.001 (35.1)	< 0.001 (36.6)	< 0.001 (36.5)	< 0.001 (21.8)	< 0.001 (25.2)
Tumour Size*	0.433 (327.5)	0.425 (328.0)	0.416 (329.0)	0.304 (340.0)	0.485 (323.0)	0.691 (307.5)	0.614 (110.5)	0.732 (106.5)
Axillary Nodal Stage**	0.501 (1.4)	0.590 (1.1)	0.494 (1.4)	0.524 (1.2)	0.792 (0.5)	0.706 (0.7)	0.708 (0.7)	0.517 (1.3)
NPI**	0.094 (4.7)	0.099 (4.6)	0.071 (5.3)	0.063 (5.5)	0.056 (5.7)	0.136 (3.9)	0.064 (5.5)	0.055 (5.7)
Histological tumour type**	0.001 (18.7)	0.001 (19.1)	0.002 (17.1)	0.003 (16.6)	0.004 (15.2)	0.004 (15.4)	0.042 (8.2)	0.051 (7.7)
LVI*	0.354 (404.5)	0.451 (394.5)	0.427 (397.0)	0.646 (378.0)	0.426 (397.0)	0.665 (376.5)	0.106 (192.0)	0.306 (174.0)
ER status*	0.001 (141.5)	0.001 (135.5)	0.004 (164.5)	0.012 (185.0)	0.002 (154.0)	0.006 (173.5)	< 0.001 (23.0)	< 0.001 (30.5)
PR status*	0.001 (186.0)	0.001 (182.0)	0.001 (185.5)	0.002 (192.0)	0.002 (192.5)	0.003 (199.0)	< 0.001 (31.0)	< 0.001 (37.0)
HER2 status*	0.006 (10.1)	0.018 (8.1)	0.001 (13.3)	0.005 (10.4)	0.004 (10.8)	0.011 (9.1)	0.013 (8.6)	0.008 (9.6)
Molecular Subtype**	< 0.001 (16.7)	< 0.001 (15.6)	0.001 (15.1)	0.002 (12.2)	0.001 (14.0)	0.005 (10.6)	< 0.001 (16.2)	< 0.001 (16.2)

AS = Average score, HS = Hot spot score. * Mann-Whitney U test, ** Kruskal-Wallis test.

Table 4: Classification of Ki67 stained sections from different tumour quadrants into low and High (at 14% cut-off point) for both the AS and HS.

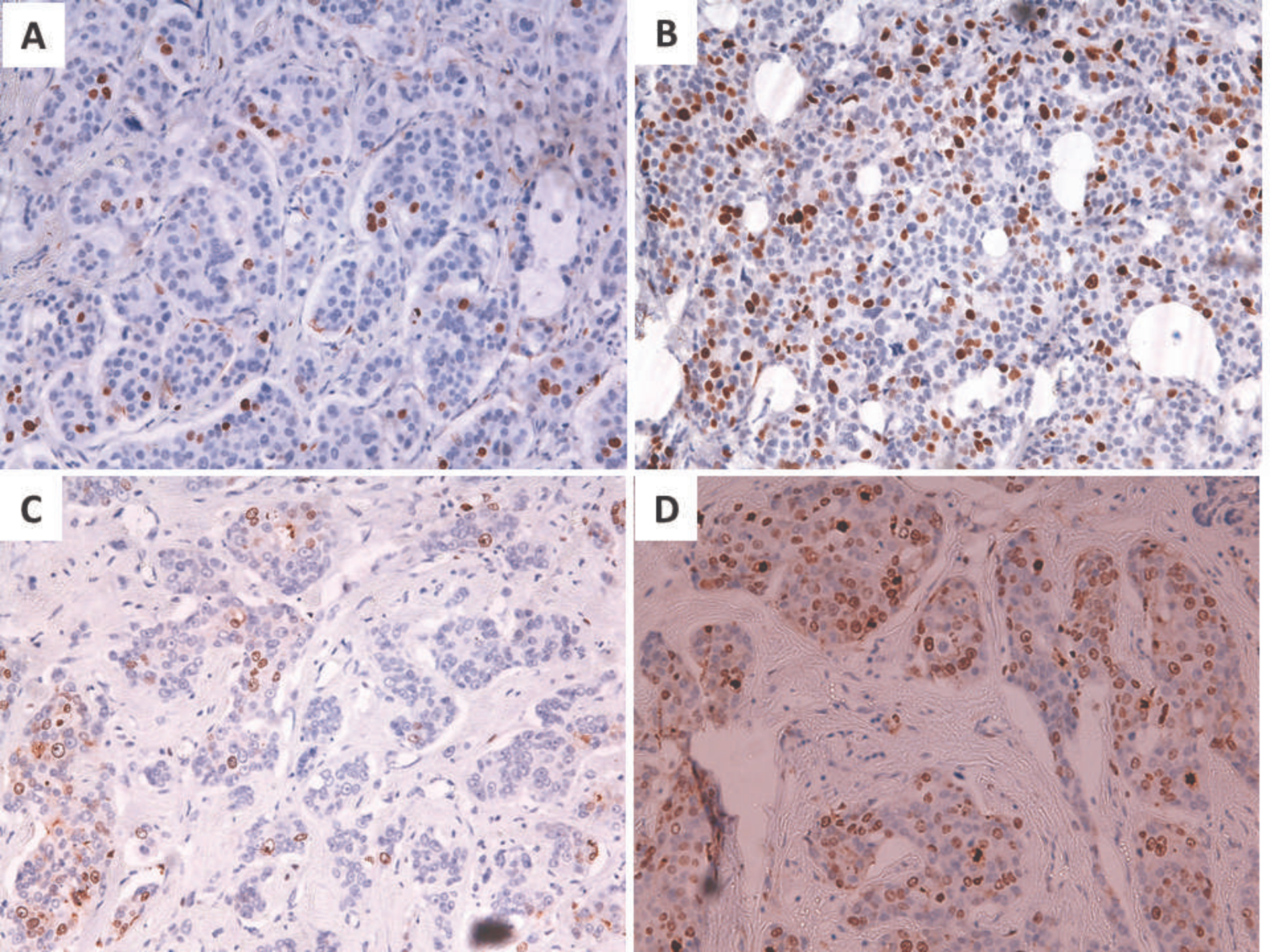
Quadrant	Ki67 HS		Total
	Low	High	
AS Quadrant 1			
Low	11	8	19
High	0	36	36
Total	11	44	55
AS Quadrant2			
Low	7	8	15
High	0	40	40
Total	7	48	55
AS Quadrant3			
Low	8	9	17
High	0	38	38
Total	8	47	55
AS Quadrant4			
Low	5	5	10
High	0	25	25
Total	5	30	35

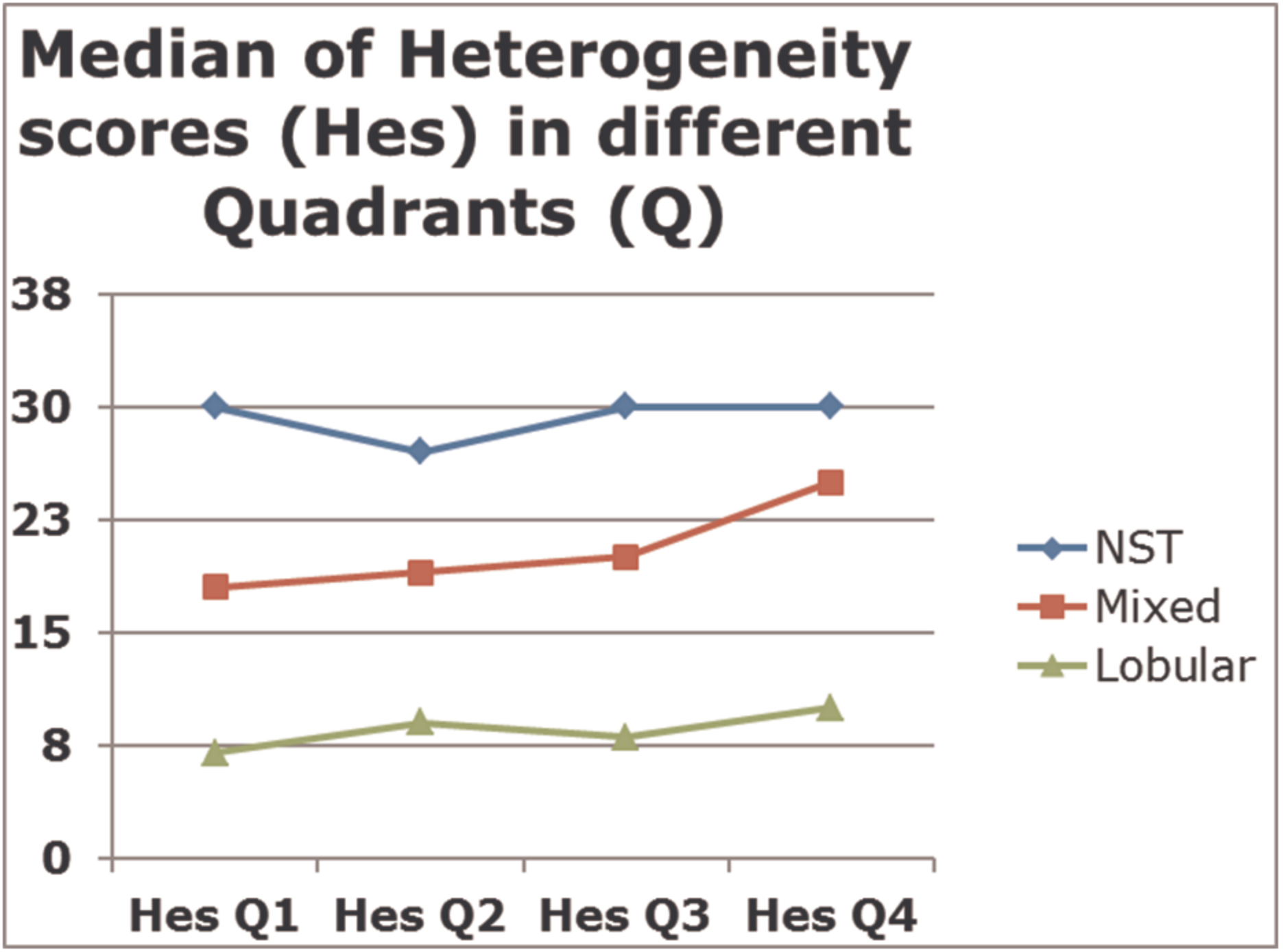
Supplementary Table 1: Summary of patient demographics of the series used in this study

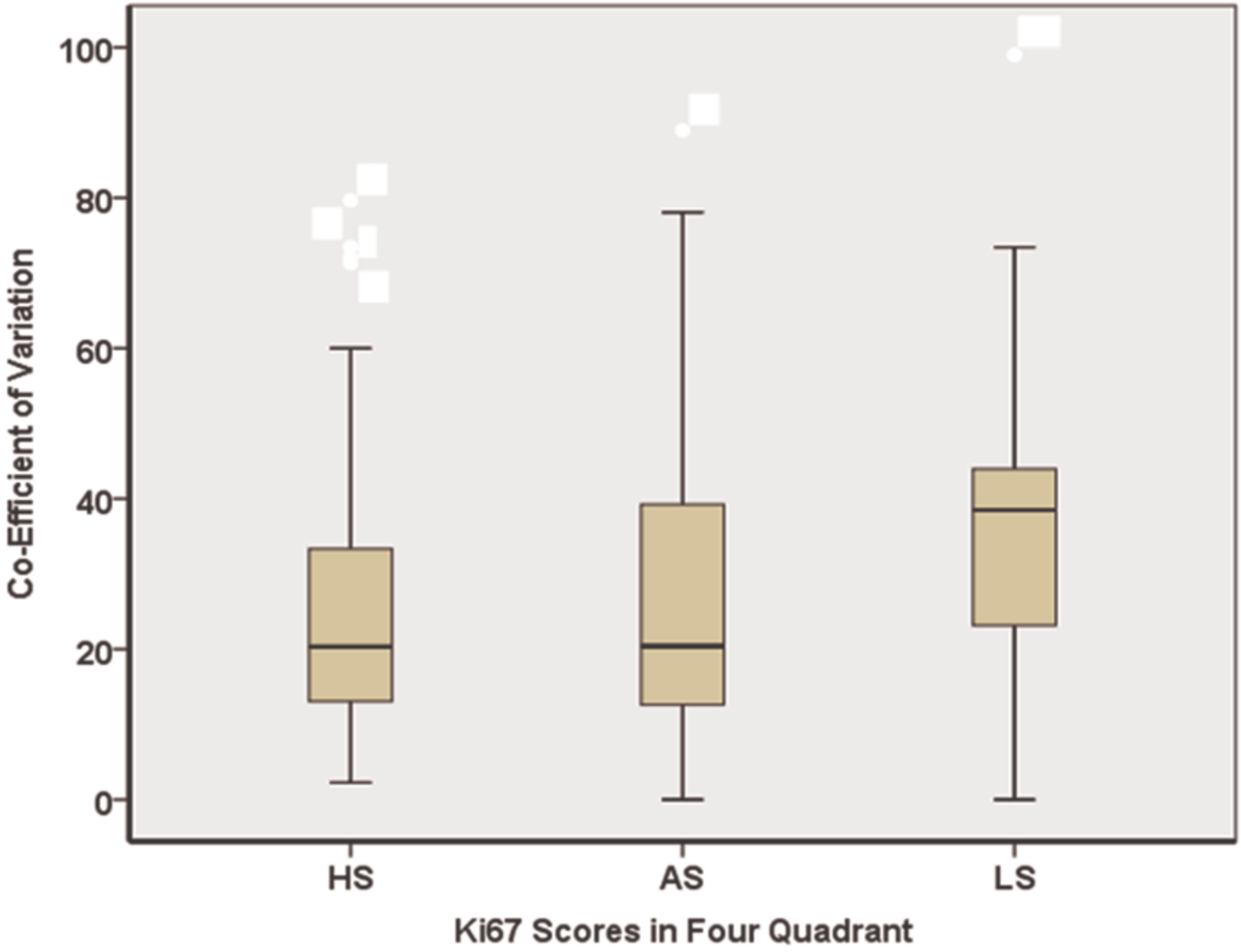
Parameter	Number (%)
Age	
≤ 50 years	25 (45.5)
> 50 years	30 (54.5)
Menopausal Status	
Premenopausal	26 (47.3)
Postmenopausal	29 (52.7)
Tumour Grade	
1	3 (5.5)
2	19 (34.5)
3	33 (60.0)
Pleomorphisms	
2	14 (25.5)
3	41 (74.5)
Tubule Formation	
2	16 (29.1)
3	39 (70.9)
Mitotic Figures	
1	16 (29.1)
2	10 (18.2)
3	29 (52.7)
Tumour Size	
≤ 2 cm	14 (25.5)
> 2 cm	41 (74.5)
Axillary Nodal Stage	
1	28 (50.9)
2	20 (36.4)
3	7 (12.7)
Nottingham Prognostic Index (NPI)	
Good NPI (<3.4)	4 (7.3)
Moderate NPI (3.41-5.4)	39 (70.9)
Poor NPI (≥5.4)	12 (21.8)
Histological tumour type	
Ductal No Special Type (NST)	44 (80.0)
Lobular*	8 (14.5)
Mixed NST and Lobular	3 (5.4)
lymphovascular invasion (LVI)	
Negative	612 (65.2)
Definite	327 (34.8)
Distant Metastasis	
Negative	32 (58.2)
Positive	23 (41.8)

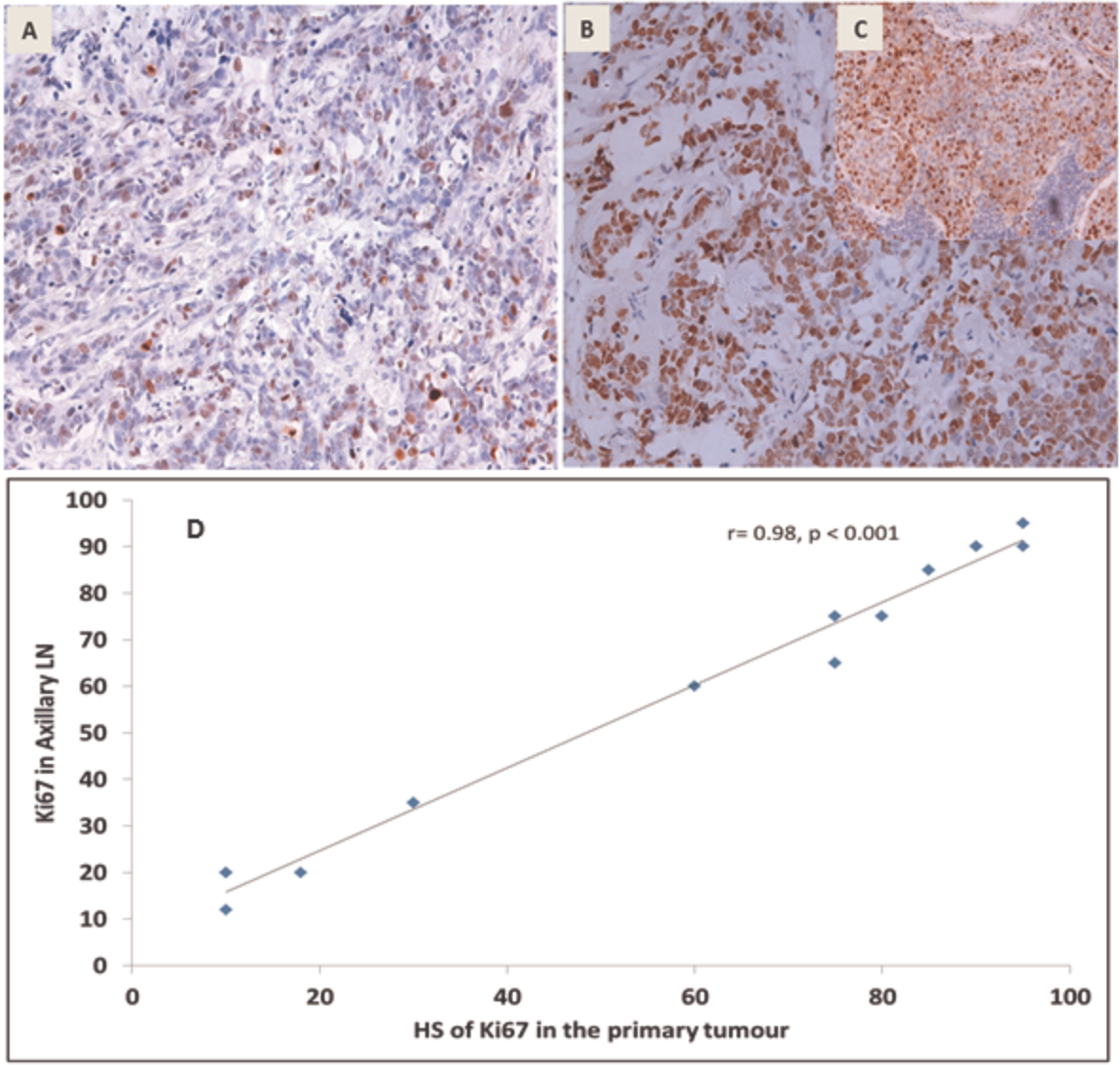
Survival (month) **Overall Survival:** Median/mean (Range) **DFI :** Median/mean (Range)	 145/131 (8-214) 104/88 (10-214)
ER status ER negative ER positive	 17 (30.9) 38 (69.1)
PR status PR negative PR positive	 26 (47.3) 29 (52.7)
HER2 status HER2 negative HER2 positive	 38 (74.5) 13 (25.5)
Molecular Subtype Luminal HER2 positive Triple Negative	 34 (63.0) 13 (24.1) 7 (13.0)

*Includes: classical lobular, alveolar lobular, solid lobular, tubule-lobular, and pleomorphic lobular.


A
B
C
D


Median of Heterogeneity scores (Hes) in different Quadrants (Q)
38
30
23
15
8
0
Hes Q1
Hes Q2
Hes Q3
Hes Q4
NST
Mixed
Lobular


100
80
60
40
20
0
Co-Efficient of Variation
HS
AS
LS
Ki67 Scores in Four Quadrant


A
B
C
D
r= 0.98, p < 0.001
Ki67 in Axillary LN
HS of Ki67 in the primary tumour
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100