Nuclear Morphometry in Prognostication of Breast Cancer in Saudi Arabian Patients: Comparison with European and African Breast Cancer

ABDELBASET BUHMEIDA[1], JAUDAH AL-MAGHRABI[2], ADNAN MERDAD[3], FATIMA AL-THUBAITY[3], ADEEL CHAUDHARY[1], MAMDOOH GARI[1], ADEL ABUZENADAH[1], YRJÖ COLLAN[4], KARI SYRJÄNEN[5] and MOHAMMED AL-QAHTANI[1]

[1]Center of Excellence in Genomic Medicine Research, [2]Department of Pathology, and [3]Department of Surgery, King Abdul-Aziz University, Jeddah, Saudi Arabia; [4]Department of Pathology, and [5]Department of Oncology and Radiotherapy, Turku University Hospital, Turku, Finland

Abstract. *Background: The role of nuclear morphometry as a prognostic factor in breast cancer is well documented. The aim of this study was to evaluate this role in breast cancer in Saudi patients and to compare it with the experience in some African and European studies. Patients and Methods: Primary tumors from 135 patients were analyzed using an image overlay drawing system (Prodit Morphometry Program), for the following nuclear features: area, perimeter, diameter, and roundness. Results: The mean nuclear area (NA) was 93 μm^2 (range 45-168 μm^2). The values of NA were higher in lymph node-positive patients than lymph node-negative patients and in advanced stages than early cancer. NA was significantly larger in patients with high grade tumor ($p<0.0001$) and in cases with tumor invasion ($p<0.01$). NA also was significantly larger in recurrent cases (103 μm^2) than in non-recurrent ones (91 μm^2). In univariate (Kaplan-Meier) analysis, NA was a significant predictor of disease-free survival (DFS) (log rank $p<0.01$), but not disease-specific survival (DSS). In multivariate (Cox) survival analysis, NA lost its significance as an independent predictor; response to treatment ($p=0.0001$) and tumor grade ($p=0.030$) being the only predictors of DFS. In a similar analysis for DSS, recurrence ($p=0.040$) and stage ($p=0.003$) were the only independent predictors. Conclusion: Nuclear morphometric profiles are helpful in identifying aggressive tumor phenotype (i.e. cases at risk for recurrence). The cut-off (93 μm^2) of NA might be applied as quantitative criterion for Saudi female breast cancer to separate patients into good and poor prognosis groups. Mean NA of Saudi patients was markedly higher than the reported mean NA in the other studies and these differences might be due to technical variations or genetic bases.*

Correspondence to: Dr. Abdelbaset Buhmeida, MD, Ph.D., Center of Excellence in Genomic Medicine Research (CEGMR). King Abdul-Aziz University. P.O. Box: 80216 Jeddah 21589, Saudi Arabia. Tel: +9662 6401000, Ext.: 25481, Fax: +9662 6952521, e-mail: abuhmeida@kau.edu.sa, abuhme@utu.fi

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Breast cancer (BC) is the most common cancer among women in western world and its incidence has increased considerably over the past decades (1-3). For example in Finland, about 3,800 females are diagnosed with breast cancer every year (4). The 5-year and 10-year relative survival rates for Finnish BC patients were 86% and 77%, respectively (5). The rise in incidence seems to depend on biological factors and also on the mammographic screening programs for detecting the cancers at an early stage. Mammographic screening programs and advanced therapies have improved the survival rates (6, 7).

According to the International Agency of Research on Cancer (IARC), BC incidence in seven African countries has doubled, from an average of 15.3 cases per 100,000 in 1976 to 33.6 per 100,000 in 1998 (8). The reason could be due to an actual increase in incidence or may be the result of improved reporting. Unfortunately, the lack of resources in poor nations in Africa makes it extremely difficult to accurately estimate BC cases in this continent. Nevertheless, the incidence of cancer, in general, appears to have increased in Africa, possibly related to the changes in social conditions, life style, and emergence of AIDS epidemic (9).

According to the Saudi Cancer Registry Report (10), BC is ranked first among females accounting for 22.4% of all newly diagnosed female cancers, with the age-specific rate of 15.4/100,000. The median age at diagnosis was 47 years (range 18-96 years).

BC is intensely studied worldwide, but many aspects still remain unclear, including the special features associated with individual countries. One of the concepts states that these geographic differences may have a genetic basis. The variation in the distribution of different genetic marker haplotypes makes this easily understandable (11). There is a clear difference between the marker haplotype distribution in western central Africa and northern Africa, and a similar difference is to be found between Asia and Europe.

The prognosis of BC can be evaluated by combining different clinico-pathological features such as tumor size, stage, grade and lymph node status (12). The histological grading system is associated with high prognostic potential (13, 14), but is still subjective, and leaves a large group of patients with unclear prognosis (15). Accurate measurements, statistically assessed, can be expected to be more reproducible than the subjective methods (16). Quantitative histopathology offers a wide range of methods for unbiased assessment, as was shown by nuclear morphometry (17-19) which was able to distinguish between benign and malignant lesions. We and others suggested that nuclear morphometry has been shown to be valuable in many countries (20, 21) and in combination with other objective prognostic criteria, can improve the evaluation of the patient's prognosis, and possibly predict response to therapy.

In the current study, the role of nuclear morphometry in the evaluation of the prognosis of Saudi BC patients was assessed, and data compared with those from Finnish (20) (European), Libyan (21) and Nigerian (22) (African) patients.

Patients and Methods

The study was performed on Saudi female BC patients, diagnosed with invasive ductal carcinoma, at the Department of Pathology, King Abdul-Aziz University, Jeddah, Saudi Arabia during years 2000-2008. The patients were excluded from this study on the basis of the following exclusion criteria: histopathological diagnosis was not invasive ductal carcinoma; patient history, and medical files, or specimens were not found. This left samples from 135 tumors available for the morphometric measurements.

The pertinent clinicopathological features (age, menopausal status, stage, grade, and lymph node status), and the follow-up and survival data were collected from patient files and summarized in Table I. The mean age at the time of diagnosis was 47.5 years (range: 19-81 years).

Treatment and follow-up. The patients were seen at 3- to 6-month intervals until death or end of follow-up (FU) which was mid-August, 2009. Some patients were lost from the FU. The mean FU time for the whole series was 47 months (range: 4-118 month). During the FU period, 25 (19%) patients developed recurrence and 19 (15%) patients developed metastasis in different organs: liver (53%), bone (26%), lung (26%), and others (10%). Disease-specific survival (DSS) and disease-free survival (DFS) were calculated as the time from diagnosis to death (due to disease) or to the date last seen alive, and time from diagnosis to the appearance of recurrent disease or date last seen disease-free, respectively. In calculating DSS, patients who died of other or unknown causes were excluded. During the FU, patients were subjected to clinical examination every 6- to 12-months and bone isotope scan, chest, and abdominal-pelvic CAT scan were performed whenever needed. In most instances, the causes of death were obvious on clinical grounds alone. Autopsy was not performed in any case.

Almost all patients were subjected to surgery in form of lumpectomy, radical or modified radical mastectomy with axillary node clearance. Postoperative early adjuvant systemic therapy in the form of chemotherapy, radiotherapy and hormonal therapy was given inclusively to 65%, 50%, and 39% of patients, respectively.

Morphometry. All tissue samples were obtained from the primary tumor at the time of diagnosis. The samples were fixed in buffered formalin and embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin. The nuclear profile of cancer nuclei was measured using Prodit Morphometry Program (Prodit 3.1, Promis Inc, Almere, the Netherlands); a digitized interactive image overlay system. The system includes a microscope, a personal computer (MultiSync 3D Color Monitor; NEC, Japan), a video camera (JVC TK-870U; JVC Japan) and digitizer board (PIP 512B video digitizer board; Matrox Electronic Systems, Dorval, Quebec, Canada). Analog images of the nuclei were outlined on the monitor screen using a computer mouse. This resulted in a digitized overlay of the traced outline. The instrument was calibrated with a micrometer slide before each measurement. Measurements were carried out at ×2500 magnification on the monitor screen (×40 objective, ×10 video ocular and ×2 internal magnification).

When examining the sections, tumor cells from the most cellular area, at the periphery of the tumor were sought. Necrotic and inflammatory areas were avoided. Averages of 10-15 microscopic fields were screened and 50 consecutive tumor cells with clear nuclear borders were outlined and measured. Overlapping nuclei were omitted. Of the morphometric variables measured by the Prodit program, the nuclear area (NA), perimeter, diameter and nuclear roundness were assessed in this study (23). To ensure reproducibility, random measurements of some samples were tested by employing two observers, and the estimations showed good correlation and reproducibility (Pearson's r: 0.89).

Statistical analysis. Statistical analyses were performed using the SPSS® (SPSS, Inc., Chicago, USA) and STATA (Stata Corp., TX, USA) software packages (SPSS for Windows, version 17.0.2 and STATA/SE 11.0). Student *t*-tests and ANOVA were used to test differences between the groups. A correlation between the morphometric parameters and survival was evaluated using Pearson's correlation test at a level of significance $p<0.05$. For univariate survival analysis, Kaplan-Meier curves were plotted, and differences between the strata (nuclear morphometric cut-offs) were analyzed using the log-rank test. In addition, we also performed multivariate analysis using Cox's regression model (with known prognostic predictors entered in stepwise approach), to evaluate the independent prognostic value of nuclear morphometry. In all analyses, *p*-values below 0.05 were regarded as significant.

Table I. *Clinico-pathological characteristics of 135 breast cancer patients.*

Characteristic	No. of patients (%)
Age (years)	
<50	89 (66%)
>50	46 (34%)
Menopausal status	
Premenopausal	90 (67%)
Postmenopausal	45 (33%)
Localization	
Right	73 (54%)
Left	62 (46%)
Margins	
Free	81 (60%)
Involved	36 (27%)
Unknown	18 (13%)
Neurovascular invasion	
No	39 (29%)
Yes	54 (40%)
Unknown	42 (31%)
Lymph node	
N0	40 (30%)
N1	67 (50%)
NX	28 (20%)
Metastasis	
M0	87 (64%)
M1	19 (15%)
MX	29 (21%)
Histological grade	
Gr I	26 (19%)
Gr II	76 (56%)
Gr III	27 (20%)
Gr X	6 (5%)
Stage	
I	23 (17%)
II	69 (51%)
III	11 (8%)
IV	17 (13%)
Recurrence during the follow-up	
Yes	25 (19%)
No	83 (61%)
Unknown	27 (20%)
Response to treatment	
CR	74 (55%)
PR	17 (13%)
PD	17 (13%)
Unknown	27 (20%)
Status at the end of follow-up	
Alive	96 (71%)
Died of disease	12 (9%)
Unknown	27 (20%)

CR, Complete response; PR, partial response; PD, progressive disease.

Table II. *Correlation of nuclear morphometry with different clinico-pathological features.*

Clinicopathological feature	*p*-Value
Age	0.22
Menopausal status	0.09
Site (R, L)	0.47
Margins	0.84
Invasions	0.01
Lymph node	0.11
Metastasis	0.09
Grade	0.0001
Stage	0.13
Response to treatment	0.06
Recurrence	0.01
DSS	0.44
DFS	0.61
Alive or not	0.07

The *p*-values refer to significant of correlation between the features listed and nuclear area.

Results

Clinicopathological features. The correlation of nuclear morphometry with different clinicopathological features is shown in Table II. The mean age at the time of diagnosis was 47.5 years. In the whole material, the mean NA was 93 μm^2 (SD=19.84).

The nuclear morphometric parameters were also analyzed in the whole material and in subgroups defined by the histological grade, clinical stage, lymph node status, lympho-vascular invasion, and recurrence. The *p*-values refer to significance of difference between the subgroups, shown in Table III. Median NA was used as the cut-off point in further calculations to correlate the NA with the clinical parameters and disease outcome. These calculations were not repeated for the other morphometric variables, because all were closely related to NA.

The NA was larger in tumors of premenopausal than postmenopausal patients ($p<0.09$). Significant associations were observed between NA and histological grade ($p<0.0001$) and tumor invasion ($p<0.01$). Similarly, a significant association was evident between the recurrence of the disease and NA, NA being significantly larger in tumors that subsequently recurred (103 μm^2) when compared with the non-recurrent ones (91 μm^2) ($p<0.01$). In the same way, NA was larger in patients, who died of their disease (104 μm^2) when compared with those who were alive at the end of the FU (93 μm^2) ($p<0.07$). NA was larger in patients who developed metastasis (101 μm^2) by the end of follow-up than in those who did not (92 μm^2) ($p<0.09$). There was also a borderline association between NA and response to treatment; complete (CR) and partial responses (PR) to

Table III. *Means of the morphometric nuclear variables (SD) in the Saudi material (n=135), and in subgroups.*

Clinico-pathological feature	Area (μm^2) (SD)	Perimeter (μm) (SD)	Diameter (μm) (SD)	Roundness (μm) (SD)
Whole material	93.00	35.48	10.79	1.05
	(19.84)	(3.79)	(1.17)	(0.02)
Histological grade	$p<0.0001$	$p<0.001$	$p<0.0001$	$p<0.88$
Grade 1	84.42	33.84	10.28	1.04
	(18.6)	(4.13)	(1.19)	(0.01)
Grade 2	93.05	35.58	10.81	1.04
	(17.08)	(3.16)	(1.00)	(0.01)
Grade 3	105.27	37.70	11.49	1.04
	(21.46)	(3.79)	(1.17)	(0.01)
Clinical stage	$p<0.13$	$p<0.27$	$p<0.18$	$p<0.09$
Stage 1	89.28	34.61	10.56	1.04
	(20.98)	(4.26)	(1.30)	(0.1)
Stage 2	95.45	35.93	10.95	1.04
	(16.43)	(3.04)	(0.95)	(0.1)
Stage 3	99.14	36.40	11.13	1.03
	(22.76)	(4.12)	(1.27)	(0.0)
Stage 4	101.07	36.79	11.20	1.04
	(28.11)	(5.14)	(1.58)	(0.1)
Lymph node status	$p<0.11$	$p<0.04$	$p<0.11$	$p<0.07$
N0	89.72	34.73	10.60	1.04
	(18.77)	(3.83)	(1.17)	(0.01)
N1	95.60	36.13	10.95	1.04
	(17.75)	(3.18)	(1.03)	(0.01)
Lympho-vascular invasion	$p<0.01$	$p<0.02$	$p<0.01$	$p<0.30$
No invasion	89.07	34.88	10.57	1.05
	(16.37)	(3.36)	(1.02)	(0.01)
Invasion	99.73	36.72	11.18	1.04
	(21.08)	(3.75)	(1.18)	(0.01)
Recurrence	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.91$
No recurrence	91.40	35.17	10.69	1.04
	(16.37)	(3.83)	(1.20)	(0.01)
Recurrence	103.12	37.39	11.36	1.04
	(21.08)	(3.92)	(1.16)	(0.01)

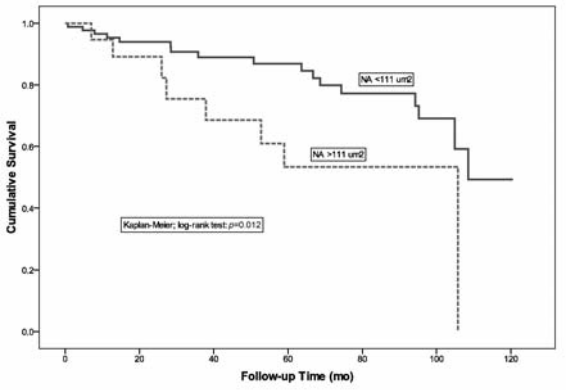

Figure 1. *NA (111 μm^2) as a predictor of disease-free survival.*

treatment were observed among patients with small NA in contrast to patients with larger NA, who developed progressive disease (PD). The mean NA of patients with CR, PR and PD were 91 μm^2, 99 μm^2, and 105 μm^2, respectively ($p<0.06$).

The values of NA were also higher in lymph node-positive patients (95 μm^2) than lymph node-negative (89 μm^2) patients, although the difference did not reach significance ($p<0.11$). The NA was larger in advanced stages (stage 3, 4) than less advanced stages (stage1, 2) ($p<0.13$). There was no relationship between age and NA, which was identical in patients below and above the mean age of 47.5 years ($p<0.22$). Similarly, NA was not related to localization of cancer either on right or left breast ($p<0.47$), and it was not associated with involvement of tumor margins ($p<0.84$).

Univariate (Kaplan-Meier) survival analysis was used to test the value of NA as a predictor of DFS and DSS. NA at cut-off (111 μm^2) was shown to be a significant predictor of DFS (log rank $p=0.012$) (Figure 1). At 5 years, 10% of patients with smaller NA showed recurrence, as compared to 44% of the patients with larger NA. Although DSS was longer (mean 96.6 month) in patients with NA below the 111 μm^2 cut-off than that (mean 71.2 months) among women with larger NA, the difference in Kaplan-Meier analysis (log-rank $p=0.189$) was not significant (Figure 2). Other morphometric parameters such as nuclear roundness neither correlated with clinicopathological features nor with disease outcome. When tested in a multivariate (Cox) model (with stepwise approach) containing the classical prognostic factors (age, family history, site, tumor grade, LNN involvement, response to treatment, stage), NA lost its significance as an independent predictor, response to treatment ($p=0.0001$) and tumor grade ($p=0.030$) being the only predictors of DFS. In a similar analysis for DSS, recurrence ($p=0.040$) and stage ($p=0.003$) were the only independent predictors. Interestingly, the mean NA of Saudi BC was much higher than the reported mean NA in the other 3 studies (20-22) (Figure 3). The range of NA in the European BC series (23-25) was smaller, varying between [24.4 μm^2 (SD: 12.8) up to 67.8 μm^2 (SD: 18.35)].

Discussion

The present study is part of our efforts to introduce a morphometric grading system specifically suitable for BC in Saudi Arabian women. We compared the present results with previously published studies from different countries (20-22), including our own conducted at the Department of Pathology, Turku University, Finland. Our present study showed a close correlation between nuclear area (NA) and different clinicopathological features and disease outcome in

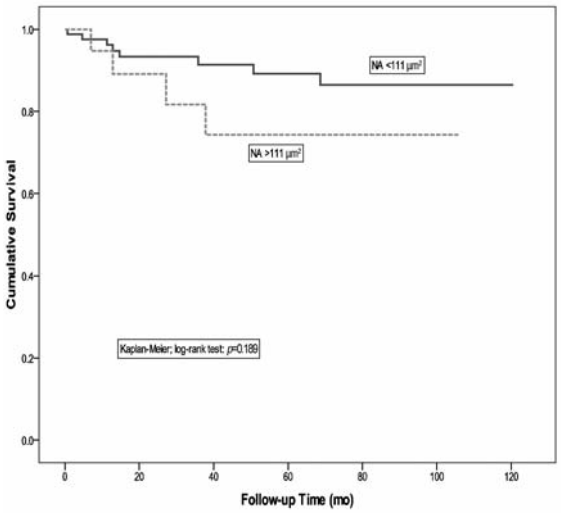

Figure 2. *NA (111 µm²) as a predictor of disease-specific survival.*

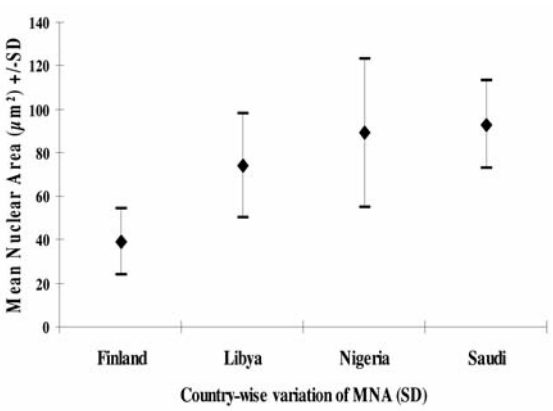

Figure 3. *Country-to-country variation of mean nuclear area (MNA) +/- standard deviation.*

BC patients from Saudi Arabia. However, the biological mechanisms responsible for these nuclear alterations in tumor cells remain to be disclosed.

The high mean MNA observed in the present series may reflect actual biological differences between BC in these populations. It is well known that significantly different tumor cell populations, clones, with dissimilar biology, exist in highly proliferating advanced breast cancers. These different clones may have different p53 status, DNA ploidy, proliferation rates and nuclear morphology (26).

There were no differences in the used morphometric methods between the four studies. The same equipment was used and the technique was standardized and uniform, with regular calibration of the computerized morphometric equipment with a micrometer slide, which should ensure reproducible results (27). However, we feel that most differences in the observed nuclear profiles among these different series (20-22) might be due to factors related to the patient materials analyzed. Baak and his colleagues (28, 29) studied the influence of delay in fixation, air-drying, acidity of 10% formalin (which is the same as 4% formaldehyde), Bouin fixative, and mercury-formalin fixatives, acetone and ethanol dehydration and under- and overstretching of the paraffin sections. They concluded that acidity had the strongest influence on NA (tested on guinea pig liver). For a pH less than 3 the NA was about 25% lower compared with formalin pH of 5-9. The pH of the fixative varied from 5 to 9, and significant differences were noted. Different fixatives gave significantly different results. The materials from Saudi Arabia, Nigeria, Libya, and Finland were not fixed with the same carefully controlled fixative. So the results are not easily comparable. This situation suggests that the results are preliminary. We cannot say what the pH of the fixative has been in each case. But we know that Nigerian fixation times have sometimes been very long. From the standpoint of fixation time, those for the Saudi, Libyan, and Finnish material are similar. However, because the fixative has not been tested, we cannot be completely sure about the influence of fixation in these different laboratories. Because of this, the results are preliminary and should be followed by a more standardized study. The results are interesting, and if true, they truly suggest that African and European (Finnish) tumors are also different from the standpoint of nuclear size. However, biological factors as explanations for these differences should not be excluded.

It is well known that computerized image analysis allows accurate and objective measurement of several nuclear features, and this technique has been used to demonstrate that increases in nuclear size and irregularity in their shape are more frequent in cancer cells than in borderline lesions (30, 31). Moreover, increasing abnormalities of nuclear morphology seem to parallel with tumor progression in various cancers, including renal cell carcinoma (32, 33), BC (34-36), and thyroid tumors (37, 38).

The independent prognostic value of nuclear variables was established in studies on infiltrating BC (39, 40). Nuclear morphometric parameters can identify an aggressive tumor phenotype and provide additional prognostic information in BC patients (41, 42).

In this study, the nuclear morphometric profiles were analyzed in the whole material and in subgroups defined by the histological grade, clinical stage, lymph node status, lymphovascular invasion, recurrence and menopausal status. Of interest was our observation that the tumors in premenopausal patients had larger NA than those in postmenopausal women. This is consistent with a Libyan

study (21) but different from the Nigerian study (22) where all nuclear measurements were significantly higher in tumors of postmenopausal women than in premenopausal patients. On the other hand, NA was useful in the premenopausal patients in the European studies (20, 43).

Of interest was also the observation that tumors with larger NA were associated with the presence of lymph node metastasis, which needs further assessment. It is likely that tumors with larger NA are more aggressive and more likely to be associated with LN involvement at diagnosis. This observation is similar to those of other studies. In agreement with this, our study showed that nuclear size features were correlated with tumor grade and stage, and this observation is similar in all three studies, being concordant with other similar cohorts (24, 44).

The mean NA in the present series (of Saudi Arabian women) was markedly higher than the reported mean NA in the other three studies (20-22) (Figure 3). The range of NA in the European BC series (23-25) was smaller, ranging between 24.4 μm^2 (SD: 12.8) and 67.8 μm^2 (SD: 18.35). The rationale behind this difference could be variations in the fixation techniques practiced in different laboratories. Another important issue is that the screening programs for BC are well established in many European countries as compared to Middle East region, which might implicate that the tumors are detected at earlier stages and more likely to be localized. The genetic difference between individual countries might also explain differences in the nuclear size variables. This analysis needs to be extended to explore the eventual biological causes behind this nuclear variability between the countries.

In the present series, the NA (with 111 μm^2 cut-off) proved to be a useful discriminator between poor and favorable DFS in univariate survival analysis. Indeed, patients with NA above 111 μm^2 showed high rate of recurrence as compared with the tumors showing smaller NA. Of the several different cut-offs tested in this study, this value was selected because it has been suggested by Ikpatt *et al*. (22) who found it useful in estimating the outcome in Nigerian BC patients. In multivariate Cox survival analysis, NA lost its significance as an independent predictor, response to treatment and tumor grade being the only predictors of DFS. In a similar analysis for DSS, recurrence and stage were the only independent predictors.

In conclusion, the present data imply that the mean NA of Saudi BC patients is markedly higher than the reported mean NA in three other studies and that nuclear morphometric profiles are helpful in identifying aggressive tumor phenotype (*i.e*. cases at risk for recurrence). We propose that morphometric measurement of NA also be applied as an objective (quantitative) criterion to distinguish BC patients into favorable and less favorable prognostic groups in Saudi Arabia.

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