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MORPHOMETRIC OBJECTIFICATION IN DETERMINING THE GRADE OF INVASIVE BREAST CANCER

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Summary

Objective: The study aimed to apply morphometric grading to cytological samples of invasive breast cancer and assess the potential for routine application, to describe the biological variability of morphometric parameters within and between grades, to optimize and determine the minimal, currently undefined, sample size required for morphometric analysis.

Subjects and methods: The study included 45 female patients diagnosed with breast cancer on cytological breast aspirates and confirmed on histological samples at the General Hospital *Dr. Josip Benčević*. Morphometric analysis was conducted using the SFORM software.

Results: The analysis of differences in the distribution of morphometric parameters by grade shows a significant, gradual increase in measured features with increasing grade. Grade accounts for approximately 35% of the variability in cell and nucleus morphometric characteristics. The majority of the variability in characteristics results from interindividual differences or heterogeneity of grade among participants, with minimal intraindividual variations. The minimal, currently undefined, sample size required for morphometric analysis was standardized to 100 cells per sample, as this ensures a narrow margin of error.

Conclusion: Grading by the morphometric method is statistically significant, but due to the low contribution of the grade to the variability of the morphometric characteristics, and the wide range of overlapping morphometric parameters among the grades, morphometry as a method does not have discriminatory but only indicative capability.

KEYWORDS: *breast cancer; fine needle aspiration cytology; morphometry*

INTRODUCTION

Breast cancer is the leading cause of death from malignancies in women, and the most frequently diagnosed cancer among women in 157 out of 185 countries worldwide(1). Aspiration cytology has been used for years as the most economical method for diagnosing breast changes(2,3). The advantages of cytological puncture lie in the fact that it is a short outpatient procedure that does not require local anesthesia, is associated with a low incidence of complications, results are quickly available, and, importantly, is very cost-effective. The disadvantages of cytological diagnosis are reflected in more inadequate samples compared to biopsy samples. To achieve accurate and reproducible results, it is crucial to implement

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quality control measures and standardize procedures from sample collection and processing to interpretation and result issuance(4,5). The term morphometry is used for various quantitative methods involving tissue and cell analyses(6,7). Numerical objectification of observed structures allows the reproducibility of the method, and, significantly, standard processed material can be used. Computer image analysis is increasingly applied in various diagnostic fields, allowing for the numerical objectification of changes in cells(8). This enables the objective quantification of parameters. Due to its quantitative features, morphometric analysis enables the correlation of tumor classification results based on morphometric data with traditional tumor classification based on subjective interpretative histopathology, with the aim of confirming that classification, increasing reliability and reproducibility of cytological and pathohistological diagnoses, potential reclassification of tumors based on morphometric data, and correlating morphometric data with prognostic indicators(9,10).

SUBJECTS AND METHODS

Based on the previous study *Morphometry of Tumor Cells in Different Types and Grades of Breast Cancer*(11), marginal distributions of the analyzed parameters were estimated to determine the required sample size for target precision and predicted statistical power (PASS program; Kaysville, Utah, USA). Patients with cytologically confirmed invasive breast cancer who underwent surgery at the General Hospital *Dr. Josip Benčević* were retrospectively selected from the medical archives of the Department of Cytology, Pathology, and Forensic Medicine at GH *Dr. Josip Benčević*, meeting two criteria: 1. existence of pathohistological verification, and 2. at least 100 preserved cells usable for morphometric analysis. The research was approved by the institutional Ethics Committee and complied with the Declaration of Helsinki. A coding system was introduced to ensure the identity of individuals whose anamnestic data was used in the research could not be determined. Informed consent was not obtained from the participants included in the study, as the data and materials were collected retrospectively from the hospital's medical archives.

Materials obtained through cytological puncture were air-dried and stained using the May-Grünwald-Giemsa (MGG) method. The determination of histological grade was conducted on resected material fixed in formalin and embedded in paraffin blocks. The currently used histologic grading system is known as the Elston-Ellis modification of the Scarff-Bloom-Richardson grading system or Nottingham Combined Histologic Grade(12). Histological characteristics used in determining the grade were degree of glandular (acinar)/tubular differentiation, nuclear pleomorphism and mitotic count(12).

Morphometric analysis was performed using the SFORM software (VamsTec, Zagreb, Croatia) with the aid of a high-resolution color Charge Coupled Device (CCD) TV camera for image transfer from the microscope (Olympus, Tokyo, Japan) to the computer. The procedure requires finding a preserved single cell, which is displayed on the monitor, and then encircled separately for the cytoplasm and nucleus using a computer mouse. The image was digitized at a resolution of 512 x 512 pixels x 24 bits. The analysis was performed on at least 100 well-preserved epithelial cells, without overlap, degenerative changes, or artifacts, at a magnification of 1000x. For each analyzed case, measurements were conducted on 100 nuclei and 100 cytoplasms, with a total of 4500 nuclei and an equal number of cytoplasms planned for a total of 45 patients (15 per grade).

The following cellular/nuclear morphometric features were analyzed:

I) Area – the region within the outlined perimeter of the cell/nucleus.
II) Contour – the shape's outline measured as the perimeter length of the cell/nucleus margin.
III) MaxR – the maximum radius, or the longest axis measured from the center to the margin within the outlined perimeter of the cell/nucleus.
IV) MinR – the minimum radius, measured as the shortest axis from the center to the margin within the outlined perimeter of the cell/nucleus.
V) Convex area – convexity; the area of a polygon described on the examined shape by tangents on its sides.

The following nuclear shape parameters were calculated based on the above-measured values:

1. AR, aspect ratio (4 x area / π x maxR x minR).
2. FF, form factor (4π x area / contour²); the degree of roundness of the object; a value of 1 indicates a perfect circle.
3. Nuclear roundness is defined as 1/AR (for round cells/nuclei, roundness values correspond to 1. If the cell/nucleus is elliptical, roundness becomes < 1).

STATISTICAL DATA ANALYSIS

Statistical analysis was conducted using the NCSS software package with a corrected two-sided significance level of <0.05. The analysis of statistical power and sample size estimation was performed using the PASS program (v07.1.14, LLC, Kaysville, Utah, USA) based on exploratory study data. The required sample size (N~100) was estimated according to the most unfavorable scenario of the exploratory study for the variable with the widest range of measured values, cell area/convex area, with a two-sided 95% confidence interval (95% CI), standard deviation (SD) 50-100, and a target margin of error of ±10 (5%) or ±15 (8%). The estimation of the statistical power of the sample was provided for the Kruskal-Wallis test, 3 equally sized groups of subjects (n=15), H0:M0=150, across a series of alternative hypotheses (M1) ranging from 150-400 arbitrary units, for SD=70, α=0.05.

RESULTS

All participants included in this study were female, with an average age at menarche of 13±1 years, a median of two childbirths (IQR 1-3; max. 8, 4 nulliparous), and an average age at menopause (33/44 participants) of 47±6.6 years. There were 14 pT1a+b and 27 pT1c+T2 tumors; 17 had axillary nodal metastases. Three participants had a positive family history of breast cancer.

The subjects were divided into three groups according to tumor grade: invasive breast carcinoma grade 1 (G1), invasive breast carcinoma grade 2 (G2), and invasive breast carcinoma grade 3 (G3).

Participants in the G2 (Δ=2.61) and G3 groups (Δ=2.15) were significantly older than those in the G1 group (p=0.03, Kruskal-Wallis test).

There is a decrease in the expression of progesterone receptors (PrR) with higher grade (p=0.027, Kruskal-Wallis test), with significantly lower PrR expression in the G3 group compared to the G1 group [3 % (0-58) *vs.* 45 % (24-69) *vs.* 60 % (60-100); G1 *vs.* G2 *vs.* G3], as well as a significant, robust, and gradual increase in the expression of the proliferation marker Ki-67 with higher grade (p=1.4x10^-6, Kruskal-Wallis test).

Menopause is a significant determinant of grade (p=0.013, Freeman-Halton test), with a significantly higher occurrence of G2 and G3 lesions in postmenopausal participants (24/30 *vs.* 3/11; p=0.003, Fisher's test).

The pT stage of the tumor is associated with the grade of the lesion: pT1a+b (<1 cm) tumors are significantly more common in the G1 group (8/15) than in the G2/3 group (6/30; p=0.039, Fisher's test). Grade 2 and 3 lesions are associated with a higher pT stage (pT1c+T2-4) at diagnosis.

No differences were observed in the expression of estrogen receptors (ER), human epidermal growth factor receptor 2 (HER2), pN status at the time of diagnosis, parity, or age at menarche between the G1, G2, and G3 lesion groups. ER expression is inversely related to the pN stage: the pN0 stage (n=29) is characterized by significantly higher ER expression [p=0.039, 95 % (80-100) *vs.* 80 % (15-94); pN0 *vs.* pN1, Mann-Whitney test]. Loss of ER expression (4/6 *vs.* 0/39; p=0.0001, Fisher's test) and PrR (3/9 *vs.* 1/35; p=0.044, Fisher's test) is associated with the pT3 stage of the tumor. ER and PrR expression strongly positively correlated with each other (ϱ=0.68, p<10^-6).

HER2 positive tumors (n=7) show significantly lower PrR expression compared to HER2-negative [0 % (0-2) *vs.* 70 % (30-98); p=0.0013, Mann-Whitney test].

No differences were found in receptor status, pT, and pN stage according to parity. The pN1 stage is associated with an earlier average age at menarche [n=15, 12 years (12-13) *vs.* 13 years (12-14); p=0.028, Mann-Whitney test]. Breastfeeding and the number of reproductive years at diagnosis were not related to any of the analyzed variables.

Analysis of differences in the distribution of morphometric parameters by grade (nested model, 15 participants per grade, 100 observations per participant, non-significant Shapiro-Wilk test) highlights a significant, gradual increase in measured features with increasing grade (Table 1).

Table 1.

Distribution of cell and nucleus morphometric parameters by grade, nested model (arithm. mean ± SD)

	Parameter	G1	G2	G3	P(G)*	% Variance (between grades)	% Variance (among respondents, within grades)
Cell	surface	190±40.2	280±71	328±71.2	<0.001	26	72
	range	57±6.1	67±8.1	73±7.8	<0.001	27	70
	minR	5.6±0.6	6.8±0.9	7.4±1	<0.001	26	70
	maxR	10.9±1.3	12.6±1.6	13.6±1.5	<0.001	21	75
	convex surface	205±42	291±73	341±72.5	<0.001	26	71
Nucleus	surface	105±18	151±35	182±41.5	<0.001	32	66
	range	40±3.4	48±5.2	52±5.7	<0.001	37	61
	minR	4.7±0.5	5.5±0.7	6.1±0.9	<0.001	24	73
	maxR	6.9±1.3	8.3±0.9	9.1±1	<0.001	41	57
	convex surface	108±18	154±35.3	186±41.7	<0.001	32	66

*F statistics (2.42)

Despite this, partitioning of variance shows that grade accounts for only 26% to 35% of the variance in morphometric features of the cell and nucleus, with the maximum contribution for nuclear maxR being 41%. The majority of variance in features is due to interindividual and intraindividual differences, i.e., heterogeneities among participants within grades and the tumors of the same participant.

The average values and intraindividual variances of all cellular parameters increase with the age of the participant at diagnosis, with no changes in cell shape measures, and no changes in the coefficients of variation of the parameters. In other words, the increase in age is accompanied by an increase in size of the cell, and the increase in size is proportionally accompanied by more pronounced anisocytosis, but without changes in the deformation pattern or the shape of the nuclei. Cell perimeter (ϱ=-0.31, p=0.042) and maxR (ϱ=-0.32, p=0.031) significantly decrease with ER expression, while area (ϱ=-0.34, p=0.021), perimeter (ϱ=-0.35, p=0.019), minR (ϱ=-0.31, p=0.036), maxR (ϱ=-0.34, p=0.024), and convex area (ϱ=-0.36, p=0.014) decrease with PrR expression. In contrast, the expression of the proliferation marker Ki-67 is strongly positively correlated with the spread of measured values (SD: $0.5<\varrho<0.6$, maxR < perimeter < area < convex area < minR, $0.000011 < p < 0.0005$), expressed by the coefficient of variation ($0.01<p<0.035$), but without affecting the average values of the parameters. Furthermore, measures of deformity are not associated with the expression of hormonal receptors. HER2 positive tumors are characterized by a significantly larger cell area [304 μm (274-354) *vs.* 230 μm (201-295), p=0.024], perimeter [72 μm (66-76) *vs.* 52 μm (57-70); p=0.026], minR [7.2 μm (6.8-7.5) *vs.* 6.2 μm (5.6-7.1); p=0.028], and maxR [13.7 μm (12.1-14.3) *vs.* 11.9 μm (10.5-13.4); p=0.028, Mann-Whitney test].

Similar patterns are observed for nuclear parameters: perimeter, area, convex area, and maxR increase with age and expression of Ki-67, and decrease with PRR expression (Table 2). MinR marginally follows the increase in age and Ki-67 expression. Neither deformity measures nor individual coefficients of variation, i.e., the dispersion of analyzed nuclear parameters, depend on the expression of ER, PrR, Ki-67, or age. Menopause is not associated with changes in morphometric variables, either of the cell or nucleus. Comparison according the pT stage shows a significant increase in the mean values of both cellular and nuclear parameters with tumor size. Nodal metastatic tumors (n=17) differ in cell shape factor [FF, 0.77 (0.75-0.78) *vs.* 0.79 (0.76-0.8), pN0 *vs.* pN1; p=0.035, Mann-Whitney test]. All cellular and nuclear micromorphometric parameters of size correlate strongly linearly with planimetric area ($p<10^{-7}$), with a somewhat more pronounced spread of the cellular maximum radius with increasing area. An exception is made for the perimeter, both, of the cell and nucleus, in terms of deviation from linearity in the highest quartile of the distribution.

Table 2.

Spearman correlation coefficients (p-values) of mean values of selected micromorphometric parameters of the nucleus and expression markers (n=45)

Parameter	Age	PrR	Ki-67
surface	0.36 (0.015)	-0.33 (0.029)	0.5 (<0.001)
range	0.36 (0.015)	-0.32 (0.031)	0.51 (<0.001)
convex surface	0.36 (0.014)	-0.32 (0.03)	0.5 (<0.001)
minR	0.35 (0.019)	NS*	0.3 (<0.001)
maxR	0.37 (0.012)	-0.36 (0.01)	0.55 (<0.001)

*NS – not significant

All morphometric parameters of the nucleus and cell are significantly associated with grade, in accordance with the results of a model based on the analysis of average parameter values. However, in combination with expression profiles and age, cell area, perimeter, and maxR do not contribute significantly or additionally to lesion classification. In contrast, representative nuclear parameters are independently associated with grade, including maxR (p=0.0002), along with the expression profile of Ki-67, PrR and age. The contribution to the improvement of classification by morphometric and expression parameters was assessed individually and collectively through discriminant analysis.

Individually, previously tested significant parameters (age, PrR, Ki-67) reduce classification error by 17%, 37%, and 50%, with a significant independent effect only of Ki-67. Among the morphometric parameters, the perimeter of the nucleus (50%), perimeter of the cell (43%), area of the nucleus (43%), and maxR of the nucleus (57%) individually most significantly contribute to the improvement of classification, with a significant yet smaller effect of the remaining cell and nuclear parameters (33-40%).

For a group of participants with a known menopausal age (n=41), postmenopausal status at the time of diagnosis reduces classification error by 27% as a variable. In combination with nuclear maxR and Ki-67, it persistently leads to an independently significant further improvement in classification (p=0.01), totaling 85%, with a trend of underestimating the actual grade in the G3 category (3/4 instances). However, caution should be taken regarding the reduced robustness and potential for generalization of this result due to the decrease in sample size.

DISCUSSION

Morphometry based on the analysis of objective parameters has proven diagnostic and prognostic value. The success of applying interactive computerized morphometry in distinguishing benign from malignant lesions, in both breast lesions(13,14) and other organ systems such as the thyroid gland(15), prostate(16), kidney(16), and liver(18) has been examined over the past two decades. The most commonly analyzed morphometric parameters are nuclear area, perimeter, convexity, maximum and minimum radius, nucleus/cytoplasm ratio, and shape factor(19-22). We performed morphometric measurements on both the nucleus and the entire cell (cytoplasm), analyzing only individual, well-preserved cells without degenerative changes or overlapping, thereby making nuclear and cellular parameters available for analysis. Tahlan et al.(23) analyzed only the cell nucleus and concluded that cytological grading significantly correlates with histological grade, with the correlation increasing upon the introduction of morphometric analysis. In their study, of all analyzed parameters, only the mean nuclear area and the mean nuclear diameter were statistically significant in predicting histological grade.

In our study, the results demonstrated the existence of significant, gradually increasing differences in the morphometric features of the nucleus and cell between different grades and proved that all morphometric parameters of the nucleus and cell are significantly associated with grade. Among the morphometric parameters applied in our research, nuclear perimeter, cell perimeter, nuclear area, and maximum radius individually significantly contribute to the improvement of classification, with a significant but smaller effect from the remaining cell and nuclear parameters. Dey et al. (24) obtained similar results in their study regarding the increase in morphometric parameter values (nuclear area, nuclear diameter, convex area, radius, and convex nuclear radius) across different grades, but they were not successful in differentiating between grades 2 and 3, which they attributed to increased heterogeneity of the cellular population in higher-grade tumors. We have also demonstrated the existence of variability within tumor cells and the variability of tumors classified in the same grade, which corresponds to biological reality given that the tumor comprises a het-

erogeneous population of cells as a result of clonal proliferation, and reengagement of the tumor. The results, therefore, indicate the extent to which cell populations branch into grades within an individual and the dispersion of cell variability within grades. Compared to the preliminary work by Prvulović et al.(11), the statistical processing of the obtained results has been upgraded, since in the preliminary work all variance was attributed to differences between different grades. Such an interpretation would only be valid if every tumor in every individual woman had an identical cell population, which we know is not the reality. The results show greater person-to-person variability within the same grade than between different grades. In other words, although there is a trend of changes across grades, cell populations are so heterogeneous and different that most variability is due to interindividual differences, not differences between grades; thus, intra-tumor variability prevails.

The above rises the question of the origin of these differences, i.e., which individual characteristics determine the extent of this dispersion. We decided to examine the influence of anthropometric variables, steroid receptor expression variables, and neoplasm proliferative markers. It is now known that ER, PrR, and HER2 status, as well as Ki-67, are excellent prognostic markers in breast cancer(25). Tamaki et al. demonstrated a significant positive correlation between Ki-67 and ER/HER2 status and histological grade. In ER-positive and HER2-negative breast cancers, Ki-67 ranged between 20% and 25%, but increased significantly with an increase in the lesion grade(26). In addition to expression markers, it is also known that age, menopause, and parity are known risk factors for the development of breast cancer. Trying to answer the question of the origin of interindividual differences, we tested a standard set of individual characteristics across all parameters for each grade. The obtained results showed that the average values and intraindividual variances of all cellular parameters increase with the age of the subjects at the time of diagnosis, but without changes in the cell shape measures. Cell perimeter and maxR significantly decrease with ER expression; similarly, area, perimeter, minR, maxR, and convex area decrease with PrR expression. In contrast, the expression of the proliferation marker Ki-67 is strongly positively correlated with the dispersion of measured values, but does not affect the average values of the parameters. Measures of deformation are not associated with the expression of hormonal receptors. HER2-positive tumors are characterized by significantly larger cell area, perimeter, minR, and maxR.

Similar patterns are observed for nuclear parameters: perimeter, convex area, and maxR increase with age and Ki-67 expression, and decrease with PrR expression. MinR marginally follows the increase with age and Ki-67 expression. Neither measures of deformation nor individual coefficients of variation, i.e., the dispersion of analyzed nuclear parameters, depend on the expression of ER, PrR, Ki-67 or age. Menopause is not associated with changes in morphometric variables, either of the cell or nucleus.

In general, we can say that a far greater degree of dispersion of individual parameters is shown by highly proliferative lesions, lesions in older patients, positive tumors, and tumors with a low level of hormone receptor expression. Although we did not find similarly designed studies in the medical literature, the obtained results align with already known facts about the biological behavior of tumors and, by themselves, do not represent new insights. However, they demonstrate the strength of the method and attest to the evidential power of the sample.

As a final consideration of the success of grading cytological smears based on measured morphometric features, it is necessary to address whether the differences in morphometric features of the nucleus and cell between individual grades are large and specific enough for reliable and unequivocal classification of smears into grades based on nucleus and cell measures.

All morphometric parameters of the nucleus and cell are significantly associated with grade. Among them, nuclear perimeter (50%), cell perimeter (43%), nuclear area (43%) and maxR of the nucleus (57%) individually most significantly contribute to the improvement of classification, with a significant, but smaller impact from the remaining parameters of the cell and nucleus (33-40%). In terms of the structure of misclassified lesions, there is a predominance of shifts to a higher category (9/13 instances).

For the group of subjects with known menopausal age, postmenopausal status at the time of

diagnosis, as a variable, reduces classification error by 27%. The fact that postmenopausal status affects the classification potential of morphometric variables indicates that the accuracy of the morphometric method in grade estimation may vary according to age or ovarian function. The same applies to other environmental factors that affect the morphometric characteristics of lesions, leading us to conclude that the applicability of morphometry may unexpectedly vary within and among populations depending on more or less (un)predictable cofactors.

It should be emphasized that the classification potential of the variables was assessed using the grading results of an experienced cytologist specializing in breast pathology. Grading can vary among observers, i.e., cytologists, so interindividual variations can produce different grading outcomes, and thus different assessments of the usability of individual morphometric parameters. Subjective differences in grading therefore limit the generalizability of the results, introducing additional uncertainty in assessing the scope of applying morphometric parameters, underscoring the need to define a gold standard in grading before analyzing the classification potential of morphometry.

The question of the sample size required for morphometric analysis still remains. Only a few authors describe the number of cells they examined in the materials and methods sections of their studies. Thus, Tahlan et al.(23) analyze in their study an arbitrary number of cells, a minimum of 50 and a maximum of 100 cells, and conclude that cytological grading significantly correlates with histological grade, with the correlation increasing with the introduction of morphometric analysis.

CONCLUSION

Grading by the morphometric method is statistically significant and there is a justification for its use, but one should keep in mind the low share of grades in the variability of morphometric characteristics (approx. < 30%), i.e., a wide range of overlapping morphometric parameters between grades, as an objective limitation of the classification potential, from which it follows that morphometrics as a method does not have a discriminating but only an indicative ability.

In our study, we determined a wide range of variations of morphometric parameters within tumors of the same person, that is, between tumors of different persons, and within the same grade, which indicates the need for further analysis of variables on independent samples of other patient populations with well-defined clinical, anamnestic and other data.

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Sažetak

MORFOMETRIJSKA OBJEKTIVIZACIJA U ODREĐIVANJU GRADUSA INVAZIVNOG KARCINOMA DOJKE

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Cilj: Cilj istraživanja bio je učiniti morfometrijsko gradiranje citoloških uzoraka invazivnog karcinoma dojke i prosuditi mogućnost rutinske primjene morfometrijske analize, umjesto dosadašnje procjene gradusa tumora na histološkom materijalu te opisati biološku varijabilnost morfometrijskih parametara unutar i između gradusa, u svrhu optimalizacije i utvrđivanja minimalne, trenutno nedefinirane, veličine uzorka potrebite za morfometrijsku analizu.

Ispitanici i metode: Istraživanjem je obuhvaćeno 45 ispitanica (15 po svakom gradusu) u kojih je dijagnoza karcinoma dojke postavljena na citološkim aspiratima dojke i potvrđena na histološkim preparatima u OB *Dr. Josip Benčević*. Morfometrijska analiza provedena je programom SFORM.

Rezultati: Analizom razlika u distribuciji morfometrijskih parametara po gradusu izdvaja se značajan, stupnjevit porast mjerenih značajki s porastom gradusa. Gradus je odgovoran za prosječno 35% varijabilnosti morfometrijskih obilježja stanice i jezgre. Glavninu varijacija obilježja čine interindividualne razlike, odnosno heterogenosti među ispitanicima unutar gradusa, uz minimalne intraindividualne varijacije. Minimalnu, trenutno nedefiniranu, veličinu uzorka potrebitu za morfometrijsku analizu normirali smo na 100 stanica po uzorku, budući da ista jamči usku marginu greške.

Zaključak: Gradiranje morfometrijskom metodom statistički je značajno, ali zbog niskog udjela gradusa u varijaciji morfometrijskih obilježja, odnosno širokog raspona preklapanja morfometrijskih parametara među gradusima, morfometrija kao metoda nema diskriminacijsku nego samo indikativnu sposobnost.

KLJUČNE RIJEČI: *karcinom dojke; citološka aspiracija tankom iglom; morfometrija*