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QUANTITATIVE ANALYSIS OF NUCLEAR VOLUME AND SIZE VARIABILITY OF BREAST SAMPLES

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ABSTRACT

Two stereological methods recently developed for the estimation of the mean volume of particles are applied to normal breast samples and to invasive ductal breast carcinomas with the aim to evaluate the efficiency of these unbiased estimates and to obtain a real size variability of the nuclei. Both volume-weighted and number-weighted mean nuclear estimates demonstrate a high capacity for differentiating between normal and pathological lesions. A good correlation between different estimates of the mean nuclear volume is found. The information concerning nuclear size variability reveals a significant increase from normal to invasive ductal breast samples when the coefficient of variation of nuclear volume $CV_N(v)$ was derived from independent estimates of number-weighted ('selector' method) and volume-weighted ('point-sampled intercepts' method) mean nuclear volume. The study shows that efficiency of the volume-weighted mean nuclear volume estimates was higher than that of the number-weighted ones.

Key words: breast, nuclear volume, point-sampled intercepts, selector, size variability.

INTRODUCTION

The application of recent advances in Stereology have demonstrated its usefulness in histopathology providing efficient and unbiased tools in discriminating normal and pathological entities as well as in grading processes (Gundersen *et al.*, 1988a, b). Their predictive power in different lesions has also been shown (Sørensen, 1989; Bundgaard *et al.*, 1992; Artacho-Pérula *et al.*, 1984). The quantitative diagnosis is mainly performed by using different estimators of nuclear size and, less often, quantitative evaluation of nuclear form. Also, several estimators of nuclear size variability are used to assist in correct diagnosis. Thus, the pathologist's subjective evaluation in association with these quantitative estimators enables the adequate classification of different lesions according to a well-designed decision process based on the combination of both subjective and objective features.

In the current study, we estimate the nuclear volume for both normal breast and invasive ductal breast carcinoma samples, and we quantify the relative variability of nuclear size (size pleomorphy). The efficiency of different estimators of the nuclear volume and the derivation of two different values for describing the coefficient of variation of nuclear volume are contrasted in this study.

MATERIALS AND METHODS

Nine normal breast and twenty-eight invasive ductal breast carcinoma samples were obtained from the files of the Pathology Service of the Reina Sofía Hospital (Córdoba, Spain). All specimens were fixed in 10% formaldehyde, routinely processed, and embedded with random orientation in paraffin wax. The retrieved paraffin blocks were newly cut for obtaining 5-μm and 25-μm thick sections which were stained with haematoxylin-eosin.

The stereological study was carried out using an Olympus BH-2 microscope modified by Bico A/S, Golstrup, Denmark, characterized by an electronic microcator (precision of vertical displacement —*z* axis— of the microscope stage: 0.5μm.), a 100 Watt halogen light bulb, and a set of motors for predetermined advance in *x* and *y* axes. An Hitachi colour camera was used for sending the microscopic image to an Ambra personal computer equipped with a Data Translation DT2851 frame grabber board. The digitized microscopic images were quantitatively measured using the modified Imago software developed by the University of Córdoba, and characterized by superposition of test systems and visualization of different microscopic images at the same time.

Sections of 5-μm thick were used for the estimation of the volume-weighted mean nuclear volume $\bar{v}_V(nucl)$(2) using the 'point-sampled intercepts' method described by Gundersen and Jensen (1985). A 100x oil immersion lens (N.A. 1.40) was used. On each systematically chosen field of vision, a rectangular counting frame and a test system composed of points associated with lines were superposed; each nucleus hit by a test point was measured in their intercept l_o and automatically registered in micrometers. Two or more intercept lengths were measured when a nucleus was hit two or more times by test points. The estimations of $\bar{v}_V(nucl)$ are obtained as follows: $\bar{v}_V(nucl) = \bar{l}_o^3 \bullet \pi/3$. The unambiguous identification of the individual nuclear profiles is the main requirement of the method. The average number of nuclear intercepts measured per sample was 119 and 125 for normal breast and invasive ductal breast carcinoma, respectively.

Sections of 25-μm thick were used for the estimation of the number-weighted mean nuclear volume $\bar{v}_N(nucl)$ using the 'selector' method (Cruz-Orive, 1987) (Fig. 1). The method is characterized in that all nuclei were sampled with the same probability, and is a combination of the 'disector' (Sterio, 1984) of unknown thickness and the previously described 'point-sampled intercepts' method. The procedure was initiated using two optical sections as a disector for sampling *n* nuclei; thus, nucleus seen in the second optical section ('reference plane'), not present in the first optical section ('look-up plane'), and captured by an unbiased frame were sampled. These nuclei were followed through all the next optical sections and in each one superposed by the test system composed of points associated with parallel lines; the intercept lengths l_o were measured through every point hitting a sampled nucleus ensuring that at least one point-sampled intercept per nucleus was obtained. Although the distances between consecutive sections do not need to be either constant or known, we have performed serial optical sections 3-μm thick using the information of the digital display of the microcator. The requirement that the distance between the first and the last section should be at least as large as the largest particle height was fulfilled in our study. Finally, the unbiased estimate of the $\bar{v}_N(nucl)$ is obtained as follows:

$$\bar{v}_N(nucl) = (\pi/3) \cdot (1/n) \cdot \sum_{i=1}^{n} \bar{l}_{o,i}^3 \qquad (1)$$

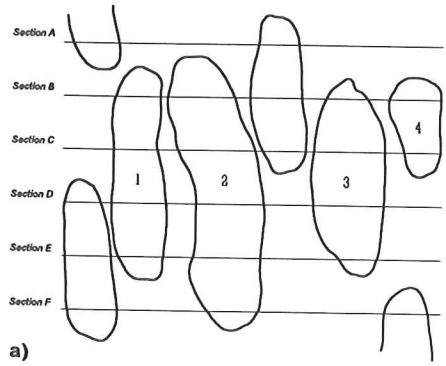
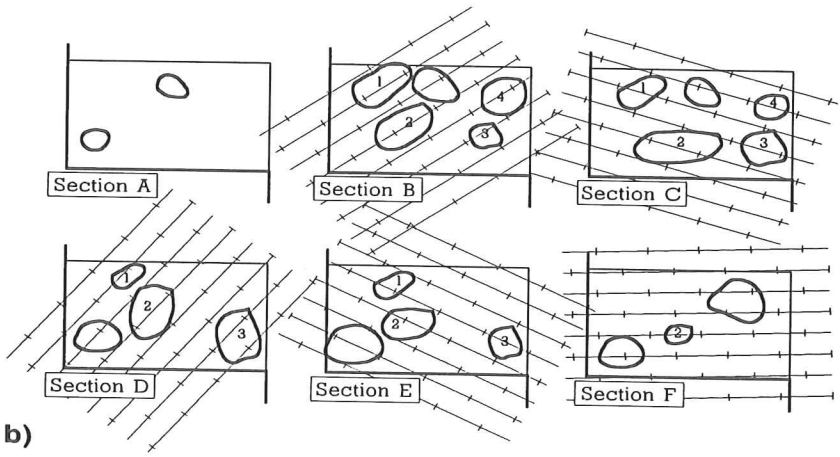


Fig. 1. Scheme showing the 'selector' method in a field of vision. a) Thick section and representative thin optical sections (A to F). b) Sections A to B are used as a disector and nuclei 1 to 4 are sampled (an unbiased counting frame is shown). From section B to F, a test system of points with associated parallel lines is superposed; the sampled nuclei are measured in their intercepts when they are hit by test points. Results are shown in Tbl. 1.



The measured intercept lengths for estimating the $\bar{v}_N(nucl)$ ('selector' method on 25-µm thick sections) are also used with the aim of obtaining the $\bar{v}_V(nucl)(1)$. This estimate is derived from the previously described equation of Gundersen and Jensen (1985) taking into consideration that only nuclei sampled by disectors were measured and these were sampled at different levels of focus. Thus, we eliminate the sampling variation in the estimates according to the paired sampling design.

The variability of nuclear size as an estimator of the size pleomorphism of the nuclear population was estimated using the $\bar{v}_N(nucl)$ values and both $\bar{v}_V(nucl)(1)$ and $\bar{v}_V(nucl)(2)$ estimates using the same or different cells, respectively. The coefficient of variation of nuclear volume $CV_N(v)$ was obtained without knowledge of the nuclear volume distribution. The calculation is as follows:

$$CV_N(v) = ((\bar{v}_V(nucl) / \bar{v}_N(nucl)) - 1)^{(1/2)} \qquad (2)$$

A self-written SAS program was used to compute the mean, standard deviation, and the coefficient of variation of each quantitative nuclear volume estimate for both normal breast and invasive ductal breast carcinomas. The same program was used for obtaining the contribution to overall observed variance of nuclear volume estimates from different levels of sampling according to the nested analysis of variance method (Gundersen and Østerby, 1981). Comparisons of normal and pathological breast samples with regard to all variables was carried out using the non-parametric Mann-Whithey test. Correlation coefficients between quantitative variables and level of significance were also obtained. Both statistical analyses were performed using the SPSS/PC$^+$ statistical package.

Table 1. Estimates of $\bar{v}_V(nucl)$, $\bar{v}_N(nucl)$ and $CV_N(v)$ corresponding to the nuclei seen in Fig. 1.

Nucleus no. i	Section B (l_o)	Section C (l_o)	Section D (l_o)	Section E (l_o)	k	$Sl_o^3{}_{,i}$	$\bar{l}_o^3{}_{,i}$
1	8.191,8.191	-	3.196	3.688	4	1181.92	295.48
2	8.191	8.627,8.627	8.409,8.409	6.670	6	3319.65	553.27
3	-	5.480	3.833,7.501	-	3	642.92	214.31
4	6.737	-	-	-	1	305.77	305.77
4					14	5450.26	1368.83

$$\bar{v}_V(nucl) = (\pi/3) \bullet (\Sigma l_o^3/\Sigma k) = (\pi/3) \bullet \bar{l}_o^3 = (\pi/3) \bullet (5450.26/14) = 407.7\ \mu m^3.$$

$$\bar{v}_N(nucl) = (\pi/3) \bullet (\bar{l}_o^3{}_{,1} + \bar{l}_o^3{}_{,2} + ... + \bar{l}_o^3{}_{,i})/i =$$

$$= (\pi/3) \bullet (\bar{l}_o^3{}_{,1} + \bar{l}_o^3{}_{,2} + \bar{l}_o^3{}_{,3} + \bar{l}_o^3{}_{,4})/4 = (\pi/3) \bullet (1368.83/4) = 358.4\ \mu m^3.$$

$$CV_N(v) = ((\bar{v}_V(nucl) / \bar{v}_N(nucl)) - 1)^{(1/2)} = ((407.7 / 358.4) - 1)^{(1/2)} = 0.37$$

RESULTS

An example of $\bar{v}_V(nucl)(1)$, $\bar{v}_N(nucl)$, and $CV_N(v)$ (1) calculations is shown in Tbl. 1.

Fig. 2 shows the results obtained for all nuclear volume estimates for both normal and pathological samples. The mean nuclear volume increases from $\bar{v}_N(nucl)$ estimates to $\bar{v}_V(nucl)(1)$ to $\bar{v}_V(nucl)(2)$ in both normal breast group ($\bar{v}_N(nucl)$: mean 108.0μm^3, SD 29.5μm^3, SEM 9.8μm^3; $\bar{v}_V(nucl)(1)$: mean 112.5μm^3, SD 30.6μm^3, SEM 10.2μm^3; $\bar{v}_V(nucl)(2)$: mean 115.9μm^3, SD 30.4μm^3, SEM 10.1μm^3) and invasive ductal breast carcinoma group ($\bar{v}_N(nucl)$: mean 383.6μm^3, SD 83.8μm^3, SEM 15.8μm^3; $\bar{v}_V(nucl)(1)$: mean 408.1μm^3, SD 85.2μm^3, SEM 16.1μm^3; $\bar{v}_V(nucl)(2)$: mean 444.3μm^3, SD 103.8μm^3, SEM 19.6μm^3). The group mean of nuclear volume in the normal breast was significantly smaller than that of the invasive ductal breast carcinoma group ($p<0.001$). Correlation coefficients for both groups were shown in Tbl. 2; a high significant correlation was found between $\bar{v}_N(nucl)$, $\bar{v}_V(nucl)(1)$, and $\bar{v}_V(nucl)(2)$. The 'point-sampled intercepts' method was

found to be five times more efficient than the 'selector' method (efficiency = 1 / (coefficient of variation • time)).

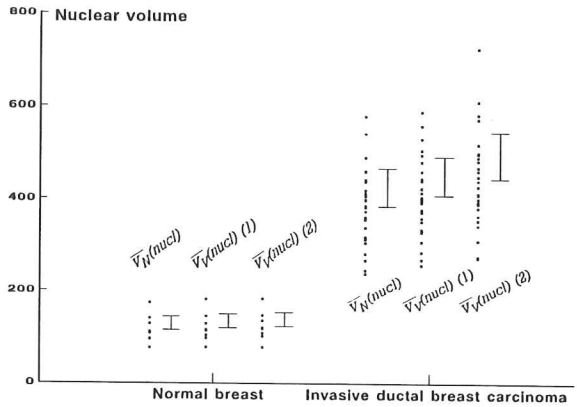


Fig. 2. Plot of nuclear volume for normal breast and invasive ductal breast carcinoma. $\bar{v}_N(nucl)$, $\bar{v}_V(nucl)(1)$ and $\bar{v}_V(nucl)(2)$ are defined in the text.

Table 2. Correlation coefficients between quantitative estimates.

		$\bar{v}_V(nucl)$ (1)	$\bar{v}_V(nucl)$ (2)	$CV_N(v)$ (1)	$CV_N(v)$ (2)
$\bar{v}_N(nucl)$	———	0.978**	0.965**	0.333	0.028
$\bar{v}_V(nucl)(1)$	0.996**	———	0.951**	-0.137	0.052
$\bar{v}_V(nucl)(2)$	0.995**	0.998**	———	-0.302	0.285
$CV_N(v)$ (1)	-0.034	0.051	0.040	———	0.039
$CV_N(v)$ (2)	-0.040	0.186	0.060	0.664	———
	$\bar{v}_N(nucl)$	$\bar{v}_V(nucl)$ (1)	$\bar{v}_V(nucl)$ (2)	$CV_N(v)$ (1)	

**Significant correlation at level of 0.01.
Bottom-left: Correlation coefficients for normal breast (n = 9). Top-right: Correlation coefficients for invasive ductal breast carcinoma (n = 28).

The results of the analysis of contribution to variance at each level of sampling is shown in Tbl. 3 for normal and pathological groups. The major contributor to the total variance is the biological variation, specially in the normal breast group. The variance due to chosen fields of vision is the smallest, excepting estimates of $\bar{v}_N(nucl)$ and $\bar{v}_V(nucl)(1)$ in the invasive ductal breast carcinoma group.

The two estimations of $CV_N(v)$ for both normal and pathological groups are plotted in Fig. 3. In both groups, the relative variation in the distribution of nuclear volume is less when the ratio between the two mean volumes ($\bar{v}_V(nucl) / \bar{v}_N(nucl)$) is calculated in the same cells.

The statistical analysis reveals significant differences between normal and pathological groups only for $CV_N(v)$ (2) (exact two-tailed p=0.001). No significant correlation between $CV_N(v)$ and nuclear volume estimators is found, or between $CV_N(v)$ (1) and $CV_N(v)$ (2).

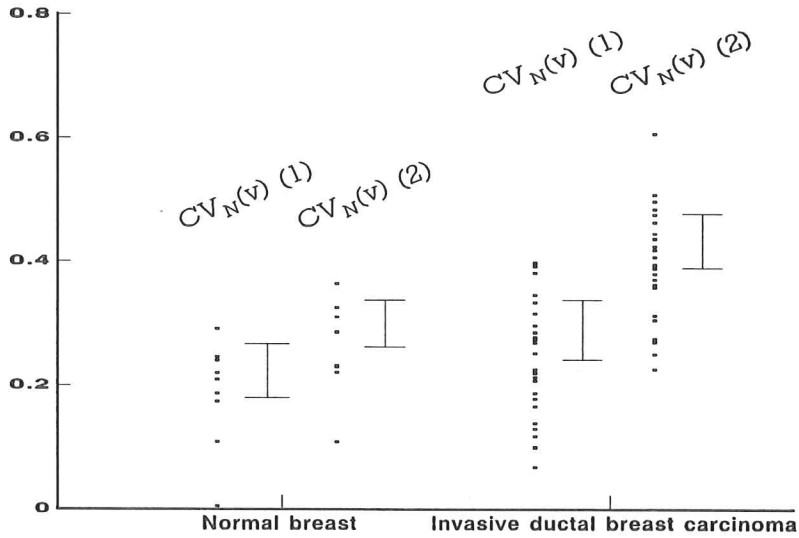


Fig.3. Plot of $CV_N(v)$ for normal breast and invasive ductal breast carcinoma. $CV_N(v)$ (1) and (2) represent the ratio between $\bar{v}_V(nucl)$ and $\bar{v}_N(nucl)$ for the same and different cells, respectively.

DISCUSSION

In histopathology, the speed in the decision process for diagnosis and/or grading of several lesions is a feature often required. Furthermore, the clinicians pursue a conclusive result of the pathologic study. These two requirements for a good treatment of patients are not fulfilled in many situations. However, the application of recent stereological methods in medicine has permitted improved histopathological classification and malignancy grading of tumors although, unfortunately, the quantitative analysis has an important disadvantage: the time consumption is greater than in the subjective, qualitative evaluations of tumor morphology. Thus, the indisputable role of the pathologist can be increased with the advantages of objective, unbiased quantitative studies.

In the histopathological context, a basic feature is the estimation of nuclear size. This nuclear characteristic could be evaluated both in terms of changes in size and of nuclear variability. The nuclear size is often obtained from two-dimensional sections resulting in two-dimensional parameters such as area, perimeter, and diameters. However, the nuclear size is more correctly defined in terms of volume. Two recently developed methods permit the obtention of unbiased nuclear volume estimates: the 'point-sampled intercepts' and the 'selector' methods. These methods are two excellent stereological tools in evaluating the mean nuclear volume, and they provide a quantitative value of nuclear size variability (size pleomorphy). This

study has demonstrated the high efficiency of the 'point-sampled intercepts' method in comparison with the 'selector' method when only measurements of nuclear volume are required. However, the selector method permits the estimation of two nuclear features (nuclear size and variability), which are often decisive in diagnosis and grading of several lesions. The results of our study have demonstrated the correct differentiation of normal and invasive ductal carcinoma of the breast using measurements of the nuclear volume. On the other hand, estimates of nuclear size variability are less useful for this purpose.

Table 3. Absolute observed variance and the relative contribution to variance at each level of sampling for the number-weighted $\bar{v}_N(nucl)$ and two estimates of the volume-weighted mean nuclear volume $\bar{v}_V(nucl)(1)$ and $\bar{v}_V(nucl)(2)$ in normal breast and invasive ductal breast carcinoma samples. Information for calculating efficiency is also included.

Parameter	Variance contribution due to:	Normal breast µm⁶ (# per biopsy)	Normal breast %	Invasive ductal breast carcinoma µm⁶ (# per biopsy)	Invasive ductal breast carcinoma %
$\bar{v}_N(nucl)$	Nuclear intercepts	90.9 (43)	9.5	1016.0 (91)	12.6
	Measured nuclei	55.9 (23)	5.8	884.9 (26)	11.0
	Fields of vision	0.0 (6)	0.0	1266.7 (6)	15.7
	Biological variation	813.0 (-)	84.7	4871.3 (-)	60.7
Total Var(est $\bar{v}_N(nucl)$ among biopsies		869.0 (9)	100.0	7022.9 (28)	100.0
$\bar{v}_V(nucl)$ (1)	Nuclear intercepts	110.4 (43)	11.8	1151.9 (91)	15.9
	Fields of vision	42.4 (6)	4.5	2605.3 (6)	35.9
	Biological variation	784.0 (-)	83.7	3499.6 (-)	48.2
Total Var(est $\bar{v}_V(nucl)$(1) among biopsies		936.8 (9)	100.0	7256.8 (28)	100.0
$\bar{v}_V(nucl)$ (2)	Nuclear intercepts	59.1 (119)	6.4	1094.2 (125)	10.2
	Fields of vision	15.3 (6)	1.6	237.1 (5)	2.2
	Biological variation	852.5 (-)	92.0	9436.7 (-)	87.6
Total Var(est $\bar{v}_V(nucl)$(2) among biopsies		926.9 (9)	100.0	10768.0 (28)	100.0

	Averaged coefficient of variation per biopsy (%)		Averaged measurement time per biopsy	Efficiency $\bar{v}_V(2)$ / $\bar{v}_V(1)$
	Normal breast	Breast carcinoma		
$\bar{v}_V(nucl)$(1)	24.3	32.3	35 min.	
$\bar{v}_V(nucl)$(2)	16.5	17.0	13 min.	4.8

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