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Objective differential classification of thyroid lesions by nuclear quantitative assessment

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Summary. Quantitative nuclear parameters estimated by morphometric and stereological methods in combination with discriminant analysis were used in order to evaluate the diagnostic efficiency of thyroid lesions. This study includes 55 patients with thyroid pathology. Samples of follicular adenomas, follicular carcinomas, and papillary carcinomas were examined by image analysis to obtain size and form nuclear parameters. Stepwise discriminant analyses were performed. There was an increase in nuclear size from follicular adenomas to follicular carcinomas, and a greater increase from follicular carcinomas to papillary carcinomas. The increase was more significant when the three-dimensional estimates of the volume-weighted mean nuclear volume were assessed. No significant differences between follicular adenomas and follicular carcinomas were found with respect to the nuclear form factors; however, a significant increase in nuclear elongation and irregularity was registered between follicular and papillary tumors ($p<0.01$). The overall accuracy rate of discrimination was 75% when the three lesions were included in the analysis, with an efficiency of 85% for papillary carcinoma samples. These percentages increased when two lesion discrimination was performed. The worst discrimination (69% of efficiency) was found between follicular adenomas and follicular carcinomas.

Key words: Thyroid lesions, Quantitative microscopy, Nuclear size, Nuclear form, Discriminant analysis

Introduction

The basis for the classification of different thyroid lesions is traditionally provided by the morphological features of the lesions in both cytological and histological specimens (La Rosa et al., 1990; Ashfaq et al., 1994). Thus, histological diagnosis of malignant thyroid lesions is effective in discriminating lesions with a papillary growth and/or an invasive pattern, but there is major difficulty in a correct diagnosis when a follicular pattern is present (Jayaram, 1985). The aid of cytology permits a more detailed cellular study, thus making diagnosis more reliable (Masuda et al., 1988). However, from a morphological point of view the differentiation between follicular adenomas and follicular carcinomas is not clear (Luck et al., 1982).

Quantitative studies have been undertaken in the search for objective diagnosis of thyroid lesions. Thus, quantitative evaluation by cytometry is of little value in distinguishing between thyroid lesions (Auer et al., 1985; Greenebaum et al., 1985; Joensuu et al., 1987), whereas the morphometric, two-dimensional characteristics of the nuclear profiles have provided relatively (and controversial) good results in differentiating benign from malignant lesions, specially in cytological studies (Boon et al., 1982; Luck et al., 1982; Wright et al., 1987). The role of the three-dimensional nuclear size estimates, i.e. nuclear volume, obtained from the recent stereological method developed by Gundersen and Jensen (1985), have been demonstrated to be useful in the diagnosis and gradation of different human tissues, but have not yet been applied to thyroid material.

The current study provides quantitative estimates in evaluating the nuclear features in three different thyroid lesions. Both nuclear size and form descriptors are reported as valuable in the diagnostic process. In addition, discriminant analyses are performed to obtain linear functions for differentiating thyroid lesions.

Materials and methods

Material

A total of 55 paraffin-embedded thyroid tissue specimens were retrieved from the Reina Sofía Hospital (Córdoba, Spain) for this retrospective study. New 5 μm-thick histological sections were obtained from the retrieved paraffin blocks and routinely stained with

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haematoxylin-eosin. The samples were categorized by two expert pathologists as follicular adenomas (FA, 20 cases), follicular carcinomas (FC, 15 cases), and papillary carcinomas (PC, 20 cases) according to the World Health Organization classification of thyroid tumors (Hedinger and Sobin, 1974).

Quantitative analysis

A Leitz Dialux 20 microscope equipped with a drawing tube in association with the digitizing tablet of a Leitz A.S.M. semi-automatic image analysis system were used for quantitative measurements (Fig. 1). Thus, each systematically selected field of vision within the section was projected onto the table by a mirror using a x40 objective lens (NA=0.70) with a final magnification of x1250. Previous calibration of the analysis system was performed by using an object microscope graticule of 10 μm equidistant lines. A cursor equipped with a light-emitting diode was used for tracing the particle (nucleus) contours on the graphic tablet. Finally, quantitative data of each particle were obtained and stored in a personal computer.

The quantitative analysis was performed by one observer experienced in the use of the system (F.B-G.), insuring that selected fields of vision corresponded to areas exhibiting features of lesion. An average of eight microscopic fields (range five to nine) were measured for each diagnosis and sample. On each projected field of vision, a graticule frame was superimposed. All nuclear profiles within the graticule were measured by outlining the nuclear boundary with the cursor of the image analysis system. Thus, the morphometric parameters corresponding to the area, perimeter, minimal diameter, maximal diameter and equivalent circle diameter of each nuclei were registered. Additionally, five form factors were calculated using

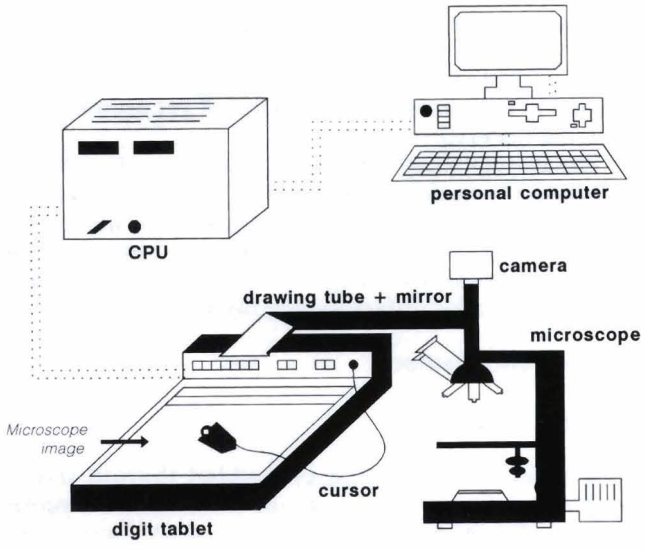


Fig. 1. Illustration of the image analysis system.

size estimators (Meijer et al., 1980; Marchevsky et al., 1987; Tosi et al., 1988) according to the following formulas: a) Form Factor PE = $4.\pi.\text{Area}/\text{Perimeter}^2$, which was 1.00 for a circle and <1.00 for elliptical and irregular structures; b) Form Factor ELL = Minimal diameter/Maximal diameter, which was 1.00 for a circle and <1.00 for elliptical structures; c) Form Factor AR = Area / ($(\pi/4)$ · Maximal diameter · Minimal diameter), which was 1.00 for a circle and an oval and <1.00 for irregular structures; d) Form Factor CO = Perimeter / $(\text{Area})^{1/2}$, which took minimum values of 3.54 for circular structures; and e) Form Factor CI = Perimeter / (π · Equivalent circle diameter), which was 1.00 for a perfect circle and >1.00 for irregular structures. The average number of measured nuclear profiles per tumour was 54, 74, and 67 for FA, FC, and PC, respectively.

On the same fields of vision, the volume-weighted mean nuclear volume *nuclear* $\overline{v}_v$ was estimated. The 'point-sampled intercepts' method described by Gundersen and Jensen (1985) was used. The *nuclear* $\overline{v}_v$ estimation was carried out superimposing a test system characterized by regularly spaced points associated with parallel lines orientated in a random direction. Each nucleus sampled (hit) by test points was measured at their intercept with the associated test line. Thus, the length l of each nuclear intercept was automatically registered in micrometers using the cursor of the analysis system when the two limits of the linear intercepts were marked (Artacho-Pérula and Roldán-Villalobos, 1994). The nuclear intercept was measured each time that a test point hit the nucleus; thus, if a nucleus was hit three times, three measurements were performed. However, the superimposed test system had spaced points that rarely hit a nucleus more than once. Finally, the *nuclear* $\overline{v}_v$ estimates were easily and unbiasedly obtained multiplying the mean of each cubed intercept length by $\pi/3$, *i.e. nuclear* $\overline{v}_v = \overline{l_o^3} \cdot \pi/3$. The unambiguous identification of the individual nuclear profiles was required. Also, sections had to be isotropic uniform random (IUR) sections, and since tissue specimens were approximately randomly orientated during routine histopathology (embedding and sectioning procedures), the condition was roughly fulfilled (Gundersen et al., 1988; Aru and Nielsen, 1989). This stereological estimator is independent of the nuclear shape and emphasizes larger rather than smaller nuclei since individual nuclei were sampled with a chance proportional to their volume. The average number of measured nuclear intercepts per tumour was 43, 60, and 62 for FA, FC, and PC, respectively.

Statistics

The mean, standard deviation, and coefficient of variation of each quantitative parameter were computed for each sample and study group using the SPSS/PC+ Statistical Software Package (DESCR procedure). A self-written SAS program (Statistical Analysis System,

Table 1. Results of quantitative data and statistical significance.

	FOLLICULAR ADENOMAS	FOLLICULAR CARCINOMAS	PAPILLARY CARCINOMAS	ANOVA		SCHEFFE TEST			KRUSKALL-WALLIS	
	(n=20)	(n=15)	(n=20)	F value	p	FA vs. FC	FA vs. PC	FC vs. PC	χ^2	p
Perimeter (μm)	24.0±8.1	25.6±3.3	29.2±4.6	10.6	<0.001	NS	<0.001	<0.05	14.3	<0.001
Minimal diameter (μm)	6.13±0.76	6.66±0.89	6.87±1.04	3.6	0.035	NS	<0.05	NS	-	-
Maximal diameter (μm)	8.29±0.69	8.73±1.07	10.0±1.49	11.9	<0.001	NS	<0.001	<0.01	14.8	<0.001
Eq. circle diameter (μm)	6.91±0.75	7.43±0.92	7.97±1.15	6.1	0.004	NS	<0.01	NS	-	-
Area (μm^2)	38.6±8.1	44.9±10.9	52.4±14.6	7.2	0.002	NS	<0.01	NS	-	-
Nuclear $\bar{v}_v$ (μm^3)	173.0±41.3	203.7±60.5	293.1±97.3	15.2	<0.001	NS	<0.001	<0.01	19.8	<0.001
Form factor PE	0.844±0.027	0.855±0.025	0.776±0.035	38.1	<0.001	NS	<0.001	<0.001	-	-
Form factor ELL	0.745±0.048	0.767±0.032	0.698±0.039	13.3	<0.001	NS	<0.001	<0.01	-	-
Form factor AR	0.957±0.018	0.961±0.012	0.934±0.026	9.4	<0.001	NS	<0.01	<0.01	13.1	<0.001
Form factor CO	3.914±0.078	3.890±0.082	4.125±0.118	34.6	<0.001	NS	<0.001	<0.001	-	-
Form factor CI	1.104±0.022	1.097±0.023	1.164±0.033	34.6	<0.001	NS	<0.001	<0.001	-	-

FA: follicular adenoma; FC follicular carcinoma; PC: papillary carcinoma; NS: not significant. Data are given as mean±standard deviation.

Inc.) was used for the three-dimensional *nuclear* v_v estimates to obtain the contribution to overall observed variance of nuclear $\bar{V}_v$ estimation from each level of sampling (nuclear intercepts within fields of vision, fields of vision within specimens, and specimens) according to the method of nested analysis of variance (Gundersen and Østerby, 1981).

One-way analysis of variance was used for comparison of all quantitative estimates in relation to three study groups (ONEWAY procedure, SPSS/PC+). Scheffe's procedure was used for two group comparisons (Scheffe, 1959). Homogeneity of variances was assessed by Cochran C statistic, whereas the Kolmogorov-Smirnov test was used for testing normal distribution (NPAR TEST K-S procedure, SPSS/PC+). In case of a lack of homogeneity of variances a non-parametric Kruskal-Wallis test was used (NPAR TEST K-W procedure, SPSS/PC+). Least-square regression was used to analyse the relationships between quantitative parameters (CORR and GLM procedures, SAS).

Several multivariant discriminant analyses were performed to optimize the differentiation between biopsies into the three study groups. A total of 33 features (including mean, standard deviation, and coefficient of variation) were included in each discriminant analysis. The SPSS/PC+ Statistical Software Package (DSC procedure) was used. Wilks' method for entering parameters into the analysis phase was used in the stepwise discriminant analysis. Finally, a classification rule using a combination of quantitative parameters was developed on cases in which group membership was known. Thus, classification function coefficients (Fisher's linear discriminant functions), discriminant scores and classification information of the cases, and the classification result table were displayed. The classification of new unknown cases can be made by using this rule with a simple equation.

Results

The quantitative data, expressed as mean and standard deviation, are given in Table 1 for follicular adenomas, follicular carcinomas, and papillary carcinomas. The nuclear size increased from follicular adenomas to follicular carcinomas (7%, 16%, and 18% for one-, two-, and three-dimensional size estimators), and from follicular to papillary carcinomas (10%, 17%, and 44% for one-, two-, and three-dimensional size estimators). Similarly, a progressive increase of the variability between biopsies with respect to the nuclear size estimators was found from follicular adenomas to follicular carcinomas to papillary carcinomas. The nuclear form factors demonstrated slight modifications between follicular adenomas and follicular carcinomas except to form factor ELL. However, the papillary carcinoma group had more nuclear irregularity and, especially, more nuclear elongation than the other two study groups.

The results of the statistical analysis of the quantitative data for the three groups is also shown in Table 1. None of the quantitative parameters demonstrated significant differences between follicular adenomas and follicular carcinomas. However, papillary carcinoma specimens showed significant differences with respect to follicular adenomas and follicular carcinomas ($p<0.01$), especially for form factors PE, CO, and CI, and nuclear $\bar{v}_v$ estimates.

Figure 2 shows the results of the analysis of the contribution of the total observed variance of *nuclear* $\bar{v}_v$ estimates for different levels of sampling. The highest level of sampling, i.e. biopsy specimens, was the main source of the total variance for the three study groups (range 65% to 83%), whereas the minor contributor corresponded to the different fields of vision selected (range 3% to 10%).

The correlation coefficients and their significance between all quantitative parameters (pooled for the three groups, n=55) is shown in Table 2. As expected, a notable, positive correlation was found between all nuclear size parameters ($p<0.001$), although the *nuclear* $\bar{v}_v$ had less correlation coefficient indexes with respect to the remaining size parameters than between the latter. A

Table 2. Correlation coefficient between quantitative parameters.

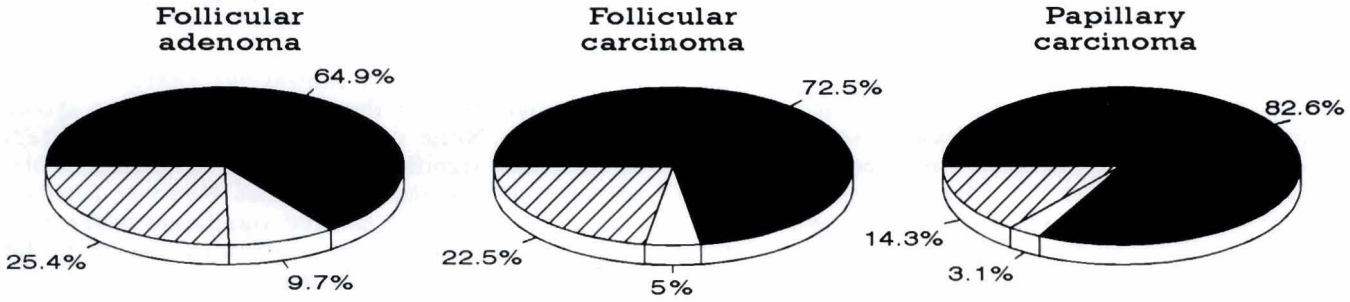
	Perimeter	Minimal diameter	Maximal diameter	Eq. circle	Area	*Nuclear* $\bar{v}_v$	FFPE	FFELL	FFAR	FFCO
Minimal diameter	0.926**									
Maximal diameter	0.982**	0.874**								
Eq. circle diameter	0.972**	0.979**	0.944**							
Area	0.980**	0.966**	0.956**	0.995**						
Nuclear $\bar{v}_v$	0.868**	0.770**	0.869**	0.828**	0.855**					
Form factor PE	-0.507**	-0.177	-0.569**	-0.313*	-0.351**	-0.493**				
Form factor ELL	-0.099	0.253	-0.239	-0.007	-0.022	-0.204	0.757**			
Form factor AR	-0.278*	-0.035	-0.400**	-0.129	-0.176	-0.364**	0.758**	0.711**		
Form factor CO	0.544**	0.227	0.596**	0.353**	0.389**	0.505**	-0.987**	-0.698**	-0.721**	
Form factor CI	0.544**	0.227	0.596**	0.353**	0.389**	0.505**	-0.987**	-0.698**	-0.721**	1.000**

*: significant correlation at level of 0.05; **: significant correlation at level of 0.01.

smaller or non-existent correlation (with positive and negative coefficients) was found between size and form parameters. Significant correlation between form factors is shown ($p<0.001$), being positive between form factors PE, ELL, and AR, and negative between these and form factors CO and CI.

Table 3 summarizes the results of the discriminant analysis when the three study groups were included in the analysis. A 75% global discrimination efficiency was reached with a correct classification of 17 out of 20 papillary carcinomas (85% of efficiency). However, seven out of 20 follicular adenomas (35% of cases), and four out of 15 follicular carcinomas (27% of cases) were misclassified. None of these erroneously classified biopsies are included as papillary carcinoma. Seven parameters were selected for this analysis with the form factor PE and *nuclear* $\bar{v}_v$ being chosen first since they had more discriminant power. The erroneously classified cases which select the correct group as a second alternative represented a percentage of 77%, whereas in only three cases the third alternative corresponded to the correct group.

The discrimination between each two groups is graphically shown in Figure 3. The difficulty in discriminating between follicular adenomas and follicular carcinomas was evident with an efficiency of 69%. However, the overall accuracy rate of discrimination between papillary carcinomas and follicular adenomas or follicular carcinomas was high (94% and 90%, respectively).



Variance contribution due to:	Follicular adenoma N	Follicular adenoma μm^6	Follicular carcinoma N	Follicular carcinoma μm^6	Papillary carcinoma N	Papillary carcinoma μm^6
Nuclear intercepts (per biopsy)	43	433	60	824	62	1357
Fields of vision (per biopsy)	8	598	8	1006	8	1645
Biological variation (biopsies)	20	1702	15	3655	20	9466

Fig. 2. Illustration of the relative percentage of contribution to the total observed variance from different levels of sampling for estimation of nuclear $\bar{v}_v$ in each thyroid lesion. Attached table: variance at each sampling level and number of measurements per item.

Table 3. Classification matrix of the stepwise discriminant program by three diagnostic groups (discriminant function coefficients are included).

DIAGNOSTIC GROUP	No. OF CASES	NUMBER OF CASES ASSIGNED TO EACH DIAGNOSTIC GROUP USING DISCRIMINANT RULES			DIAGNOSTIC AGREEMENT (%)
		FA	FC	PC	
Follicular adenoma	20	13	7	-	65%
Follicular carcinoma	15	4	11	-	73%
Papillary carcinoma	20	2	1	17	85%
Total % agreement					75%

DISCRIMINANT FUNCTIONS	FA	FC	PC	WILKS' LAMBDA	EQUIVALENT F STATISTIC
Constant	-769.4	-778.1	-702.1		
FFPE	1612	1618	1521	0.405	38.1
Nuclear $\bar{v}_v$	0.181	0.183	0.210	0.334	18.6
DMIN (SD)	-152.1	-126.0	-146.1	0.302	13.7
Area (CV)	-0.953	-0.208	-0.211	0.267	11.5
DMIN (CV)	16.4	14.5	15.1	0.250	9.6
Area (SD)	3.81	2.73	3.25	0.230	8.5
FFELL (SD)	74.2	60.9	76.2	0.217	7.5

FA: follicular adenoma; FC: follicular carcinoma; PC: papillary carcinoma; FFPE: form factor PE; DMIN: minimal diameter; FFELL: form factor ELL; (SD) and (CV): standard deviation and coefficient of variation of parameter.

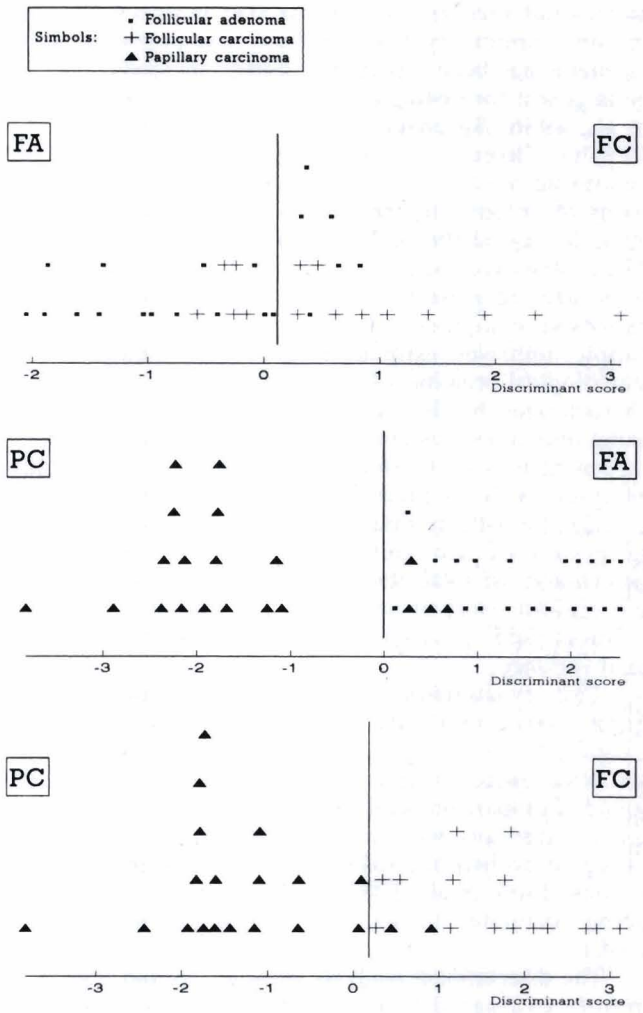


Fig. 3. Plots of the results of discriminant analyses with respect to two-group comparison of thyroid lesions. FA: Follicular adenoma; FC: Follicular carcinoma; PC: Papillary carcinoma.

Discussion

Quantitative studies in pathology have led to improvements in the accuracy and precision of the subjective diagnoses made in routine histopathology. The additional, objective information gained through quantitation of nuclear morphological features in combination with multivariate statistical techniques may be useful in the correct classification of different lesions. Thus, its application in discriminating between benign and malignant lesions as well as in the grading process have been widely reported (Paplanus and Graham, 1987; Baak, 1991; Artacho-Pérula et al., 1994a). Similarly, the application of three-dimensional reconstruction in microscopy permits us to examine the structure itself and the spatial quantitation (Salisbury, 1994), in both neoplastic and non-neoplastic lesions. However, this methodology is complex and expensive, and the application of stereological principles permits the knowledge of average spatial relationships based on theoretical grounds.

The results of this investigation confirm the utility of the nuclear size and form assessments in distinguishing between thyroid lesions. There was an increase in nuclear size from benign to malignant follicular tumors with a more evident increase when papillary carcinomas were assessed. Similar results were previously reported (Luck et al., 1982; La Rosa et al., 1990). However, the quantitative assessment of the follicular tumors of the thyroid has not provided a clear differentiation between both benign and malignant lesions while papillary carcinomas are rarely misinterpreted with respect to follicular neoplasms. These results are in concordance with others (Luck et al., 1982; Paplanus and Graham, 1987) whereas Boon et al. (1980) reported a notably, significant increase in nuclear area from follicular adenomas to follicular carcinomas. Perhaps, this discrepancy may be due to the material used (histological and cytological material respectively). However, La Rosa et al. (1990) found results similar to ours in the cytological evaluation of thyroid samples, reporting a significant, although low, differentiation between follicular adenomas and carcinomas ($p<0.04$) when nuclear area was examined.

The nuclear size measurement obtained by the three-dimensional *nuclear* $\bar{v}_v$ estimates described a more

porcentual increase in nuclear size between thyroid lesions, specially for papillary carcinomas. This estimator has been reported in numerous studies with a great power for distinguishing different lesions (Nielsen et al., 1986; Sørensen, 1989; Artacho-Pérula et al., 1994b). Three main advantages are present when measuring *nuclear* $\overline{v}_v$: i) description of nuclear size in terms of volume; ii) independence of any assumptions about nuclear shape; and iii) efficiency of the procedure. Thus, objective methods to estimate nuclear size and form are, in general, time consuming and require expensive equipment. However, the development of a simple, unbiased estimation of nuclear volume using stereological principles (Gundersen and Jensen, 1985) is characterized by the easy estimation of this parameter using only a test system, a ruler, and a pocket calculator (although it is preferable, if possible, to use an image analyser) with no excessive time consumption (on the average, ten-fifteen minutes per biopsy). In addition, the precision of the method is adequate with high percentages of total observed variation associated with the highest level of sampling, i.e. biopsies, whereas different fields of vision are the minor contributor to the total variance.

The evaluation of the nuclear shape using quantitative methods demonstrated more nuclear irregularity and elongation in papillary carcinomas. No statistical differences between follicular lesions were found. Comparison with other studies is difficult since these estimators were not reported, due to either not being of additional value in distinguishing thyroid lesions (Luck et al., 1982; La Rosa et al., 1990) or to being correlated to the nuclear area (La Rosa et al., 1990).

The discriminant analysis between thyroid lesions is mainly evaluated in terms of efficiency. Also, the number of variables selected and the probability of both correct or incorrect classification of biopsies are useful in evaluating the discriminant power of the analysis. Thus, the global efficiency of discrimination between the three groups is relatively high (75% correctly classified) using eight parameters, and 65% of the misclassified cases had more than a 30% probability of being correctly classified. The papillary carcinomas are the most correctly classified biopsies (85% of cases). Global efficiencies of 90% and 94% are reached when two group discrimination (follicular adenomas versus papillary carcinomas, and follicular carcinomas versus papillary carcinomas, respectively) is performed. However, no increase in two group discrimination efficiency was found between follicular lesions, although the number of variables chosen (four) was smaller. These results are in agreement with the general difficulty in separating follicular lesions and the correct diagnosis of papillary carcinomas. Ferrer-Roca et al. (1990) performed discriminant analysis on thyroid samples using flow cytometric and densitometric methods in combination with morphometric parameters. To complicate comparisons with our results, thyroid lesions were differently grouped. A notable discrimination (97% of the overall accuracy rate) was shown between adenomas and carcinomas (including follicular, papillary, mixed, and undifferentiated types) using ten parameters (one corresponding to nuclear size).

In conclusion, we underline the value of nuclear form factors and nuclear size, especially the three-dimensional *nuclear* $\overline{v}_v$ estimates, for improving patient diagnosis of the thyroid neoplasms. However, benign and malignant follicular tumors do not show a clear differentiation using quantitative nuclear estimates. The combination of several quantitative features increases discrimination efficiency, mainly associated with papillary carcinomas.

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