Use of morphometry in cytological preparations for diagnosing follicular non-Hodgkin’s lymphomas

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SUMMARY Morphometric techniques were applied to cytocentrifuge smears from 27 patients with histologically confirmed follicular non-Hodgkin’s lymphoma to determine the usefulness of this method in diagnosis. Analysis of quantitative data confirmed that most subtypes were classified correctly on the basis of the proportion of large cells, small cleaved, and large non-cleaved cells, and nuclear, nucleolar, and cytoplasmic features.

Morphometry is a more objective and reproducible technique than manual cell counting methods, and the use of cytocentrifuge smears has several advantages compared with conventional histological sectioning. Data derived with the aid of morphometry may be of value in follow up clinicopathological studies.

The Working Formulation for Clinical Usage separates follicular or nodular lymphomas into three distinct morphological subtypes.1 Follicular, predominantly small cleaved cell, and mixed (small cleaved and large cell) are low grade tumours, sensitive to chemotherapy, but associated with short lived complete remission. Patients are often managed by adopting a “watch and see” approach.2 Follicular mixed lymphoma is often characterised by a more aggressive clinical course than its counterparts within the low grade category, however, and aggressive treatment with curative intent is controversial.3,4 Conversely, more labile clinical features, including a shorter mean survival, are a feature of follicular, predominantly large cell lymphoma, and this subtype is classified by the Working Formulation to be of intermediate grade. According to the Working Formulation, patients with intermediate grade lymphoma are usually treated with multiagent chemotherapy regimens on initial presentation.

Clearly, there are degrees of low, intermediate, and high grade lymphoma, and the proper management of patients, with appropriate choice of treatment regimens, relies on adequate clinical assessment and correct pathological diagnosis.5 The difficulties which pathologists experience in providing a reproducible subjective evaluation was highlighted in a recent study of follicular lymphomas,6 with major disagreement evident in 37% of cases. The authors of this study emphasised the inherent difficulties in making a correct diagnosis including determination of the proportion of large cells, classification of cells into small, intermediate, and large size categories, and differentiation between cleaved and non-cleaved cells.

To assist pathologists in the categorisation of these lymphomas more objective methods including the manual estimation method of Berard6 and semiautomated techniques6 have been proposed. Dar-dick et al analysed morphometrically derived data, obtained on the basis of nuclear variables in histological sections from 54 cases of follicular centre cell lymphoma, to determine whether subclassification could be achieved using this technique.8

To further assess the usefulness of morphometric techniques in the classification of these lymphomas we extended the study to incorporate not only nuclear data but quantitative information relating to nucleolar and cytoplasmic variables. Furthermore, to minimise problems inherent in quantitative studies of histological sections, semiautomated image analysis was performed on cytocentrifuge preparations, obtained from cell suspensions of representative lymphoid tissue.

Material and methods

Cytological specimens from patients with histologically confirmed non-Hodgkin’s lymphoma were analysed. The histological diagnosis was made in
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acCORDANCE with the Working Formulation for Clinical Usage and the Rappaport scheme. They included seven cases of follicular, predominantly small cleaved cell (nodular, poorly differentiated lymphocytic) (NPDL); nine cases of follicular, mixed small cleaved and large cell (nodular, mixed lymphocytic—“histiocytic”) (NM); six cases of diffuse, predominantly large cell (DL) (diffuse “histiocytic”) (DH); and five cases of follicular, large cell (FL) (nodular “histiocytic”) (NH). For the purposes of this study, cases of diffuse and follicular, predominantly large cell lymphomas (DL + FL), were combined with those of diffuse and nodular “histiocytic” (DH + NH).

Histological diagnosis was made by an experienced pathologist of this specialty (KSC).

Fresh lymphoid tissue was forwarded to the laboratory with minimal delay, and before fixation was divided for histological, immunological, and ultrastructural studies. A representative sample of tissue, measuring 5–10 mm³ was collected into RPMI 1640 and cytological smears were prepared as previously described. Briefly, cell suspensions were obtained by gently teasing lymphoid tissue through a 1 mm gauge wire mesh. The cell suspension was resuspended in 30% bovine serum albumin and cytological smears were prepared with the cytocentrifuge (Shandon Scientific Ltd, London). The centrifuge was spun at 250 rpm for five minutes and the preparation was air dried before staining with Jenner’s giemsa.

Random non-overlapping fields of the cell preparation were photographed on 35 mm film and the photographic negatives were projected by Leitz projector on to a digitiser pad (HP9874A). The nuclear, nucleolar, and cytoplasmic contours of all lymphoid cells within the field, excluding those with disrupted cell membranes, were traced. The morphometric features and statistical variables (table 1) were processed by a Hewlett-Packard 9000 (series 200) computer, interfaced with the digitiser pad.

To determine the optimal number of cells to be analysed, computer generated plots of the cumulative mean of nuclear area were derived for each case; a minimum of 150 cells were digitised and the measurement procedure was completed when the fluctuation in the cumulative mean was within 5% over at least 50 separate measurements. The total number of cells traced for each case ranged from 150–300 cells.

Quantitation of nuclear shape was achieved using a shape descriptor previously applied in the field of powder technology. Convexity-concavity (C-C) was used as it has a number of advantages over conventional shape indices, such as the nuclear contour index (NCI)-divergent irregular shaped nuclear profiles are not assigned similar index values.

For the purposes of this study, a cleaved nucleus was defined as one possessing one or more invaginations where the margins of the invagination were parallel, or the angle subtended by the walls of the invagination was less than 30°. Only those invaginations showing clear separation of contours were traced, as we have found poor reproducibility associated with digitising linear grooves or infoldings of the nuclear membrane (unpublished data). A nucleus with an assigned index value of less than 0.9 was classified as cleaved.

To exclude normal mature lymphocytes from the cell population, a total of 250 cells present in six cases of non-Hodgkin’s lymphoma were traced; these cells were selected on the basis of morphological criteria. A mean nuclear area of 36.4 μm² was obtained for the sample and the upper 95% confidence interval of 37.6 μm² was chosen as the cut off point for exclusion of these morphologically benign cells. Likewise, a similar approach was followed to select the threshold value for nuclear shape. Hence all small regular shaped cells with a nuclear area of <37.6 μm² and a C–C of ≥0.9 were excluded from study; exclusion of these cells was a continuous process during the collection of data.

Separation of lymphocytes into small and large cells was achieved by comparing the nuclear size of lymphoma cells with that of the tissue macrophage, a comparison that often assists pathologists in making this distinction. The nuclear contours of 100 macrophages, selected on the basis of cytological criteria, were traced. A mean nuclear area of 133 μm² was obtained for the population and the lower 95% confidence interval of 87 μm² was chosen as the cut off point for separation of small and large cells.

Using nuclear size as a criterion, subdivision of nodular lymphomas into follicular small cleaved cell (FSC), follicular mixed cell (FM), and diffuse and follicular large cell (DL + FL) was made when the proportion of large cells was less than 20%, between 20–50%, and greater than 50%, respectively.

Further separation of the cell population into categories representing the main types of follicular centre cells was achieved using data on nuclear area and shape. Computer generated scatterplots were

<table>
<thead>
<tr>
<th>Feature</th>
<th>Statistical variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear area</td>
<td>Mean</td>
</tr>
<tr>
<td>Nuclear shape*</td>
<td>SD</td>
</tr>
<tr>
<td>Nucleolar area</td>
<td>Skewness (skew)</td>
</tr>
<tr>
<td>Nucleolar: nuclear ratio †</td>
<td>Kurtosis</td>
</tr>
<tr>
<td>No nucleoli/nucleus</td>
<td>25th percentile</td>
</tr>
<tr>
<td>Cytoplasmic area</td>
<td>75th percentile</td>
</tr>
<tr>
<td>Nuclear: cytoplasmic ratio</td>
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</tbody>
</table>

*Mean feret diameter/perimeter equivalent diameter, †area of all nucleoli per cell/area of nucleus‡; all statistics listed calculated for each feature.

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derived for each case and the relative proportions of small cleaved, large cleaved, and large non-cleaved cells were calculated using the threshold values previously mentioned.

Inter- and intraobserver variability were assessed for the cell variables under investigation. Reproducibility was within 5% for all variables with the exception of nucleolar area, which showed variability in individual measurements of less than 10%.

Multiple comparisons for the morphometric features and statistics studied between the three subtypes of nodular lymphoma was performed by one way analysis of variance (ANOVA). Where significant differences were shown to be present by the variance F-test (p < 0.05), the results were compared by the Newman-Keuls test.15 Significance was set at p < 0.05 and all differences referred to in the text met this criteria.

Results

Table 2 lists those morphometric features and statistics that show significant differences (p < 0.05) between subtypes. Table 3 shows the mean (SEM) for the raw data.

Analysis of the results provided useful information regarding classification of these groups. Separation of FSC compared with DL + FL and FM compared with DL + FL was achieved using several variables; fewer differences were noted between FSC and FM lymphomas. This finding was related to the varying proportions of large cells that comprised each subtype. Fig 1 depicts the relation between histological diagnosis and the percentage of large lymphoid cells determined by morphometry, using the threshold values defined by Warnke et al.14 On the basis of this criterion two cases each of FSC, FM, and DL + FL were classified as FM, DL + FL, and FM, respectively. Moreover, there was a significant difference

<table>
<thead>
<tr>
<th>Feature</th>
<th>Follicular small cleaved cell v follicular mixed cell (nodular poorly-differentiated v nodular mixed)</th>
<th>Follicular small cleaved cell v diffuse and follicular large cell (nodular poorly-differentiated v diffuse and follicular large cell)</th>
<th>Follicular mixed cell v diffuse and follicular large cell (nodular mixed v diffuse and follicular large cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear area (μm²)</td>
<td>SD, Mean</td>
<td>Mean, SD, 25th, 75th</td>
<td>Mean, SD, 25th, 75th, Mean</td>
</tr>
<tr>
<td>% large cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear shape</td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
</tr>
<tr>
<td>% Small cleaved</td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
</tr>
<tr>
<td>% Large non-cleaved</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear area (μm²)</td>
<td>Skewness, Kurt</td>
<td>Mean, SD, 25th, 75th</td>
<td>Mean, 25th, 75th</td>
</tr>
<tr>
<td>Nucleolar/nuclear ratio</td>
<td>SD, Skewness</td>
<td>Mean, SD, 25th, 75th</td>
<td>Mean, 25th, 75th</td>
</tr>
<tr>
<td>Cytoplasmic area (μm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear:cytoplasmic ratio</td>
<td></td>
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</tbody>
</table>

Fig 1  Distribution of proportion of large cells (nuclear area > 87 μm²) for each case grouped according to lymphoma subtype.
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Table 3  Morphometric data of follicular lymphoma*

<table>
<thead>
<tr>
<th>Feature</th>
<th>Follicular small cleaved cell (nodular poorly differentiated lymphocytic) (n = 7)</th>
<th>Follicular mixed cell (nodular mixed cell) (n = 9)</th>
<th>Diffuse and follicular large cell (diffuse and nodular &quot;histiocytic&quot;) (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear area (µm²)</td>
<td>Mean: 63.0 (2.0)  SD: 17.9 (1.7)  Skewness: 0.831 (0.194)  Kurtosis: 2.0 (0.945)  25th: 50.9 (2.0)  75th: 71.8 (2.4)  % Large cells: 14.6 (3.2)  Nuclear shape: % small cleaved: 10.8 (2.7)  % large non-cleaved: 11.6 (2.8)  % large cleaved: 3.0 (0.769)</td>
<td>Mean: 76.7 (3.2)  SD: 30.7 (2.5)  Skewness: 1.3 (0.198)  Kurtosis: 3.0 (2.6)  25th: 55.2 (2.1)  75th: 92.0 (5.3)  % Large cells: 34.8 (4.6)  Nuclear shape: % small cleaved: 4.3 (1.5)  % large non-cleaved: 26.8 (2.4)  % large cleaved: 8.1 (2.3)</td>
<td>Mean: 105.9 (5.8)  SD: 43.6 (3.6)  Skewness: 1.3 (0.198)  Kurtosis: 0.789 (0.462)  25th: 72.7 (6.3)  75th: 132.9 (7.2)  % Large cells: 65.1 (4.9)  Nuclear shape: % small cleaved: 0.991 (0.515)  % large non-cleaved: 53.8 (4.1)  % large cleaved: 11.3 (3.4)</td>
</tr>
<tr>
<td>Nucleolar area (µm²)</td>
<td>Mean: 1.4 (0.056)  SD: 0.452 (0.045)  Skewness: 1.4 (0.231)  Kurtosis: 3.5 (0.794)  25th: 1.1 (0.944)  75th: 1.6 (0.660)</td>
<td>Mean: 1.7 (0.167)  SD: 0.719 (0.184)  Skewness: 2.5 (0.238)  Kurtosis: 10.4 (1.6)  25th: 1.3 (0.070)  75th: 1.9 (0.208)</td>
<td>Mean: 2.4 (0.193)  SD: 1.2 (0.214)  Skewness: 2.2 (0.240)  Kurtosis: 7.7 (1.8)  25th: 1.7 (0.109)  75th: 2.7 (0.206)</td>
</tr>
<tr>
<td>Nucleolar: nuclear ratio</td>
<td>Mean: 0.023 (0.001)  SD: 0.008 (0.001)  Skewness: 1.1 (0.097)  Kurtosis: 1.4 (0.273)  25th: 0.017 (0.001)  75th: 0.026 (0.001)</td>
<td>Mean: 0.025 (0.002)  SD: 0.011 (0.001)  Skewness: 1.6 (0.257)  Kurtosis: 4.5 (1.8)  25th: 0.018 (0.001)  75th: 0.030 (0.002)</td>
<td>Mean: 0.031 (0.002)  SD: 0.014 (0.001)  Skewness: 1.2 (0.253)  Kurtosis: 2.5 (1.6)  25th: 0.020 (0.001)  75th: 0.039 (0.002)</td>
</tr>
<tr>
<td>No nucleoli/nucleus</td>
<td>Mean: 0.615 (0.047)</td>
<td>Mean: 0.819 (0.055)</td>
<td>Mean: 1.0 (0.065)</td>
</tr>
<tr>
<td>Cytoplasmic area (µm²)</td>
<td>Mean: 74.0 (3.0)  SD: 27.8 (3.0)  Skewness: 1.3 (0.218)  Kurtosis: 3.1 (1.6)  25th: 54.9 (2.5)  75th: 87.5 (4.2)</td>
<td>Mean: 95.2 (5.2)  SD: 52.8 (4.9)  Skewness: 1.9 (0.259)  Kurtosis: 6.3 (1.6)  25th: 60.0 (2.4)  75th: 115.1 (7.8)</td>
<td>Mean: 132.3 (8.2)  SD: 64.3 (5.8)  Skewness: 0.720 (0.169)  Kurtosis: 0.714 (0.380)  25th: 85.4 (8.5)  75th: 169.6 (10.5)</td>
</tr>
<tr>
<td>Nucleolar: cytoplasmic ratio</td>
<td>Mean: 0.820 (0.015)</td>
<td>Mean: 0.793 (0.011)</td>
<td>Mean: 0.792 (0.010)</td>
</tr>
<tr>
<td>SD</td>
<td>Mean: 0.932 (0.005)</td>
<td>Mean: 0.114 (0.005)</td>
<td>Mean: 0.102 (0.003)</td>
</tr>
<tr>
<td>Skewness</td>
<td>Mean: 0.932 (0.005)</td>
<td>Mean: 0.114 (0.005)</td>
<td>Mean: 0.102 (0.003)</td>
</tr>
<tr>
<td>Kurtosis</td>
<td>Mean: 0.574 (0.060)</td>
<td>Mean: 0.188 (0.067)</td>
<td>Mean: 0.077 (0.019)</td>
</tr>
<tr>
<td>25th</td>
<td>Mean: 0.764 (0.021)</td>
<td>Mean: 0.715 (0.018)</td>
<td>Mean: 0.724 (0.012)</td>
</tr>
<tr>
<td>75th</td>
<td>Mean: 0.893 (0.013)</td>
<td>Mean: 0.885 (0.008)</td>
<td>Mean: 0.870 (0.010)</td>
</tr>
</tbody>
</table>

*Mean (SEM).

between the mean percentage of large cells present in each subtype.

Separation of FSC compared with DL + FL and FM compared with DL + FL was achieved with most of the statistical variables analysed for nuclear area. The large cell lymphomas (DL + FL) were characterised by a range of cell types with a predominance of cells with nuclear area measurements of > 87 µm²; hence cell distributions were typified by higher values for mean, 75th percentile (fig 2), standard deviation, 25th percentile, and with a lower value for kurtosis, suggesting a relatively flat distribution, although there was no significant difference between subtypes for this statistic.

Follicular mixed cell lymphomas occupy an intermediate position between FSC and DL + FL lymphomas, except for skewness and kurtosis, which reflect nuclear area distributions more positively skewed, and with a more pronounced peak with respect to FSC and DL + FL lymphomas. Follicular, small cleaved lymphomas comprise a monotonous population of small cells, and standard deviation of nuclear area was of value in differentiating this subtype from FM and DL + FL lymphomas (fig 3).

Nuclear shape was useful in differentiating groups, and fig 4 depicts the proportion of small cleaved, large non-cleaved, and large cleaved cells within each subtype. The percentage of small cleaved cells was significantly different between FSC compared with FM and between FSC compared with DL + FL, with FSC lymphomas comprising a greater proportion (10% (SEM) 2.7%), of these cells. No significant differences were noted between FM and DL + FL subtypes nor was the proportion of large cleaved cells of value in differentiating groups.

Greater differentiation was achieved by analysing the proportion of large non-cleaved cells within each subtype and a trend towards greater numbers of large
Fig 2  Distribution of mean and 75th percentile of nuclear area, as determined for each case and subtype of non-Hodgkin's lymphoma. (Mean SEM).

As with nuclear area, fewer differences were noted between FSC and FM lymphomas with respect to nucleolar data. The shape of the distribution, however, as described by skewness and kurtosis, was significantly different. Follicular mixed cell lymphomas were characterised by nucleolar distributions that were more positively skewed in favour of larger nucleoli and with a more pronounced peak, suggesting a greater incidence of both small and large nucleoli.

Differentiation of subtypes was also achieved with measurements of cytoplasmic area and results were similar to those obtained for nuclear area. Differences between groups were again related to the varying proportions of small and large cells. Nuclear: cytoplasmic ratio was unhelpful in classifying subtypes, but the standard deviation of this feature separated FSC and FM lymphomas, as large cells with more abundant cytoplasm are a feature of FM lymphomas.

Discussion

The results of our study confirm that morphometry is a useful adjunct in categorising follicular lymphomas,
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mixed cell lymphomas occupying an intermediate position. Considerable overlap between groups was discerned. Additional quantitative data can be derived, however, from nuclear area measurements and the lymphoid population can be separated into small and large cells by using the nuclear dimensions of tissue macrophages, which provides more meaningful information than pooled mean data. Using the percentage of large cells as a criterion, two cases each of FSC, FM, and DL + FL lymphoma were incorrectly classified.

We agree with Dardick et al. that case to case evaluation of follicular centre cell lymphoma is important and that this approach allows the pathologist to identify the borderline cases. Moreover, the threshold values chosen for separation of subtypes may be refined in further studies which relate quantitative cell data to clinical variables including survival.

Nuclear shape data derived in this study were not directly comparable with the findings of previous investigations as we used a new shape descriptor for the quantitation of nuclear shape. The shape index (C–C) is specific for invaginations where the nuclear contour exhibits an abrupt deviation, as seen with cleaved profiles, and is less influenced by the overall

although more differences were noted between FSC compared with DL + FL and between FM compared with DL + FL than between FSC and FM subtypes. Differences between groups were related to the varying proportions of large cells within each subtype, and quantitative data derived for nuclear, nucleolar, and cytoplasmic features substantiate these findings. Quantitation of the large cell component is important as recent cell kinetic studies suggest that the proportion of these cells may determine responsiveness to chemotherapy, with subsequent prolonged disease free survival.

The distribution of nuclear area measurements for cases in our study was similar to that obtained by Dardick et al., who concluded that on the basis of morphological criteria follicular lymphomas represent a continuum of one disease process, with nodular

Fig 4 Distribution of proportion of cell types, as determined by nuclear area and shape data for each case and subtype. (Mean SEM).

Fig 5 Distribution of mean and 75th percentile of nucleolar area, as determined for each case and subtype of non-Hodgkin's lymphoma. (Mean SEM).
shape of a nucleus as is the case with conventional shape indices such as the NCI.12

Computer algorithms were devised to partition cells into distinct categories on the basis of nuclear size, and shape and information regarding the proportion of small cleaved, large cleaved, and large non-cleaved cells was derived. These morphological terms were originally proposed in the classification of Lukes and Collins18 and recently incorporated in the Working Formulation.1 As with nuclear area data we considered this approach to provide more useful information than pooled data.

Analysis of the proportion of cell types within each subtype (fig 4) provided some interesting observations. There was a trend towards fewer numbers of small cleaved cells in diffuse and follicular large cell lymphomas with follicular small cleaved cell comprising the highest percentage of this cell type. About 10% of cells in this subtype possessed nuclear invaginations, which met the criteria for classification as cleaved profiles. It must be emphasised that only nuclear invaginations, where intervening cytoplasm was discerned, were traced; unacceptable inter- and intra-observer reproducibility was associated with digitising linear grooves or infoldings of the nuclear membrane.

The proportion of small cleaved cells has been shown to be underestimated on histological sections of patients with follicular small cleaved cell lymphomas. On the basis of computer modelling studies, the 4–5% of cells classified as cleaved on sections actually represent 25–30% of the cell population.9 Our value of 10% small cleaved cells is slightly higher than the 4–5% calculated by Dardick, as the cytocentrifuge technique tends to accentuate subtle variations in nuclear shape,9 and tangentially sectioned profiles would be expected to display less morphological detail than cells flattened on to a glass slide.19 This value is still not an accurate representation of the small cell component.

The proportion of large non-cleaved cells differed significantly between subtypes with an expected trend towards greater numbers of these cells in diffuse and follicular large cell lymphomas. Likewise, a similar trend was noted for large cleaved cells, although these cells comprised a smaller percentage of the large cell population. No significant difference in the proportion of these cells was discerned.

Small non-cleaved cells were not analysed in this study as tumours composed predominantly of this cell type almost invariably present as a diffuse pattern. Follicular lymphomas of this cell type have been described.1 The nuclear dimensions of these cells approximate to that of a tissue macrophage, and further studies are required to formulate appropriate computer algorithms to differentiate these cells from the large non-cleaved population. Quantitative data relating to mean nucleolar area, number of nucleoli per nucleus, and location of nucleoli within a nucleus may be of value in making this distinction. Furthermore, the upper and lower confidence limits, derived from the nuclear area of tissue macrophages, could be selected as threshold values to define the size range for this cell.

The importance of nucleolar data as a prognostic indicator is unclear, although nucleoli are identified in those cells which have been known to have an adverse influence on survival. Nucleolar area distributions (table 2, fig 5) show features similar to those obtained for nuclear area with follicular small cleaved cell lymphomas, characterised by fewer and smaller nucleoli, ranging to greater numbers of larger nucleoli in the diffuse and follicular large cell subtype. Whether the case of nodular mixed cell lymphoma, typified by a greater percentage of large cells with more prominent nucleoli, follows a more unfavourable clinical course will need to be determined in follow up clinicopathological studies.

Manual counting of cell types in follicular centre cell lymphomas forms the basis of the standard and modified Berard methods, and this more objective assessment of the percentages of each cell type has been found to be of value in classifying these lymphomas.6–7 None the less a certain degree of subjective interpretation is associated with these methods, including the distinction between small and large cells and the classification of cells on the basis of nuclear shape.

Morphometry is an objective and reproducible method that may be of value to the pathologist in classifying this disease entity. Moreover, the technique provides quantitative data which may predict clinical outcome, may select those patients who require more aggressive treatment, and may allow for more meaningful comparisons of the results of clinicopathological trials between centres.

We thank Ms Erica Fairbanks for preparation of the illustrations.

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7 Nathwani BN, Metter GE, Miller TP. What should be the morphologic criteria for the subdivision of follicular lymphomas? Blood 1986;68:837–45.


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