

# Morphometric characterisation of 52 B cell non-Hodgkin's lymphomas

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**SUMMARY** Fifty-two B cell non-Hodgkin's lymphomas, in which the diagnosis was based on immunological, cytochemical, and ultrastructural studies, were characterised by morphometry on plastic-embedded tissue sections.

Parameters studied were: nuclear size, cytoplasmic area, cytoplasm to nucleus ratio, nuclear contour index, nucleolar size, location of nucleoli within the nucleus, as expressed by relative nucleolar eccentricity, and the mean number of nucleoli per nuclear cross-section. The results of the measurements and subsequent statistical analysis show that the different types of lymphoma can be distinguished from each other, even though the differences were small. Small cell lymphomas (chronic lymphocytic leukaemia, lymphoplasmacytoid and polymorphic immunocytoma, centrocytic, centroblastic/centrocytic and intermediate lymphocytic lymphomas) could be separated from each other by the standard error of mean (SEM) of nuclear area, the cytoplasm to nucleus ratio, and nuclear contour index. Large cell lymphomas (centroblastic and B immunoblastic lymphomas) could be differentiated with cytoplasm to nucleus ratio and nucleolar parameters (relative nucleolar eccentricity and number of nucleoli per nuclear cross-section). The morphometric parameters of lymphoblastic lymphomas fell in the range of the large cell lymphomas, but lower SEM indicate these lymphomas were more monomorphic. These morphometric data underline the concept of the Kiel classification and establish the usefulness of morphometry as an additional technique in diagnosis.

Non-Hodgkin's lymphomas morphologically and immunologically from a heterogeneous group. Differentiation in subclasses is important as it clearly has clinical relevance.<sup>1-3</sup> The distinction between large cell and small cell lymphomas is relatively easy, but differentiation of subtypes within these two groups is difficult. The differences are small and subtle and are easily obscured. Additional techniques such as marker studies, enzyme histochemistry, and electron microscopy are therefore employed to support the morphological diagnosis. Morphometric measurements of the nuclei is a further way in which this may be substantiated.

In this study we have used morphometry on methyl-methacrylate-embedded lymph node sections to characterise 52 B cell non-Hodgkin's lymphomas, of which the diagnosis was confirmed previously by marker studies, enzyme histochemistry, and electron microscopy. The results show that definite

morphometric differences can be found between the subclasses of the small cell and of the large cell lymphomas.

## Material and methods

### MATERIAL

Fifty-two B cell non-Hodgkin's lymphomas were studied. In diagnosing the lymphomas the Kiel classification<sup>4</sup> was followed as modified by Lennert.<sup>1</sup> Furthermore, a recently recognised entity, the intermediate lymphocytic lymphoma,<sup>5</sup> was studied. The distribution was as follows:

- Chronic lymphocytic leukaemia (CLL): 5 cases.
- Lymphoplasmacytoid immunocytoma (LP): 5 cases.
- Polymorphic immunocytoma (PI): 5 cases.
- Centrocytic lymphoma (CC): 5 cases.
- Centroblastic/centrocytic lymphoma (CB/CC): 5 cases.
- Centroblastic lymphoma (CB): 9 cases.
- Intermediate lymphocytic lymphoma (ILL): 5 cases.

- B immunoblastic lymphoma (IB): 8 cases.
- Lymphoblastic lymphoma, Burkitt type (LB): 5 cases.

All cases were diffuse proliferations, except the cases of centrocytic and centroblastic/centrocytic lymphomas, that showed nodularity.

## METHODS

### *Tissue processing*

Each tissue specimen was cut into four pieces. From one of these four pieces, approximately eight blocks (1 mm<sup>3</sup>) were cut for electron microscopy. The blocks were fixed in a fixative according to McDowell and Trump.<sup>6</sup> One quarter was fixed in buffered formalin for paraplast embedding; one was fixed in a sublimate-formaldehyde mixture<sup>7</sup> and subsequently embedded in paraplast for immunohistochemistry. One piece was fixed in a fixative according to Burkhardt<sup>8</sup> for methyl-methacrylate embedding and subsequent morphometry, and the fourth piece was snap-frozen in liquid nitrogen for immunofluorescence, rosette assays and enzyme histochemistry.

### *Histology*

Paraplast sections (4 µm) were cut and stained with haematoxylin and eosin, Giemsa, PAS with and without diastase treatment, and a reticulin stain. Methyl-methacrylate sections were cut at 2 µm and stained with Gallamine-Giemsa, PAS and Gomori's reticulin stain.

### *Immunohistochemistry and -fluorescence*

Immunohistochemistry was used to stain for intracytoplasmic immunoglobulins (kappa, lambda, A, M, G, D, and E), lysozyme, alpha-1-antitrypsin and alpha-1-antichymotrypsin. An indirect immunoperoxidase method was used on 4 µm paraplast-embedded sections. The antisera and their specificity were described extensively earlier by us.<sup>9</sup> Immunofluorescence, employed to stain for membrane-bound immunoglobulins, was performed on 4 µm cryostat sections, also using an indirect technique, using the same antisera as for immunohistochemistry. Control studies were conducted according to Sternberger.<sup>10</sup>

### *Rosette assays*

Presence of receptors for the activated third component of complement and the Fc portion of IgG (C3 and Fc gamma receptors) was tested using the red-cell overlay technique<sup>11 12</sup> using ox erythrocytes coated with rabbit IgM antibodies and mouse complement, or ox erythrocytes coated with rabbit

IgG antibodies. Control studies were performed using IgM coated or uncoated ox erythrocytes.

### *Enzyme histochemistry*

Staining was carried out on 8 µm cryostat sections. Enzymes stained for were 5'-nucleotidase and adenosine triphosphatase<sup>13</sup> (with adenosine monophosphate and triphosphate as respective substrates at pH 7.2), acid phosphatase and alpha-naphthyl acetate esterase<sup>14</sup> (with β-sodium glycerophosphate (pH 5.0) and alpha-naphthyl acetate (pH 7.2) as respective substrates) and alkaline phosphatase<sup>15</sup> (with β-sodium glycerophosphate as a substrate at pH 9.6). Counterstaining was done with haematoxylin.

## ELECTRON MICROSCOPY

After fixation the tissue blocks were postfixed in osmium tetroxide, dehydrated in a graded series of alcohol solutions and embedded in Epon. Ultrathin sections were cut on a LKB III microtome and stained with uranyl acetate and lead citrate. The sections were examined in a Siemens Elmiskop 1a or a Philips 201C electron microscope.

All techniques have been described previously.<sup>9</sup>

## MARKER PATTERN USED TO DELINEATE B CELL LYMPHOMAS IN TISSUE SECTIONS

- Chronic lymphocytic leukaemia (CLL):
  - presence of monoclonal surface immunoglobulins (sIg);
  - absence of monoclonal intracytoplasmic immunoglobulins (cIg), weak C3 receptors.
- Lymphoplasmacytoid immunocytoma (LP) and polymorphic immunocytoma (PI):
  - presence of monoclonal sIg and cIg, but weak to absent C3 receptors.
- Centrocytic lymphoma (CC):
  - presence of monoclonal sIg, absence of monoclonal cIg and strong C3 receptors.
- Centroblastic/centrocytic lymphoma (CB/CC):
  - presence of monoclonal sIg, absence of monoclonal cIg and strong C3 receptors.
- Centroblastic lymphoma (CB):
  - presence of monoclonal sIg, absence or near-absence of monoclonal cIg, moderate to strong C3 receptors.
- Intermediate lymphocytic lymphoma (ILL):
  - presence of monoclonal sIg, absence of monoclonal cIg, moderate C3 receptors.
- B immunoblastic lymphoma (IB):
  - presence of monoclonal sIg and cIg, weak to absent C3 receptors.
- Lymphoblastic lymphoma, Burkitt type (LB):
  - presence of monoclonal sIg, absence of monoclonal cIg, weak C3 receptors.

Electron microscopy was useful to distinguish between the large cell lymphomas. Centroblastic lymphomas had scarce rough endoplasmic reticulum, whereas B immunoblastic lymphomas had ample rough endoplasmic reticulum.

#### Morphometry

Methyl-methacrylate embedded, Giemsa-stained sections were used. Photographs were made of 7–10 characteristic fields of the tumour, selected by two experienced, independent pathologists (final magnification of the photographs was  $\times 1700$ ). All cells on the photograph having clear nuclear outlines were numbered and measured. The measurements were made by tracing the outline of a nucleus on a graphic tablet interfaced to a PDP 11/10 minicomputer. The computer printed out area, perimeter and nuclear contour index (NCI) of each measured nucleus. The NCI is a size-independent shape parameter, defined as  $\frac{\text{perimeter}}{\sqrt{\text{area}}}$ .

Its minimal value is found for a circle and is 3.545. It increases when the nuclear irregularity increases.<sup>17,18</sup> From each case 100–120 cells were measured and mean nuclear area, mean cytoplasmic area, mean cytoplasm to nucleus ratio, mean NCI and their SEM were calculated.

Nucleolar parameters—that is, the size of the nucleoli (area), their location within the nucleus, and the mean number of nucleoli per nuclear cross-section, were determined in the cases of centroblastic lymphoma and B immunoblastic lymphoma, also using a graphic tablet. In most cases nucleoli were so large and conspicuous that they were easily identified. In some cases however, fixation had been suboptimal and nucleoli had to be distinguished from clumps of chromatin, caused by suboptimal fixation. All accumulations of chromatin with surface area  $> 0.5 \mu\text{m}^2$ , of uniform density and a more or less regular contour were regarded as nucleoli. Nucleoli and nuclei were traced with a cursor and the computer (MOP-AMO<sub>3</sub>, Kontron) calculated the size of both nucleolus and nucleus, as well as their respective centres of gravity, expressed in co-ordinates in an X-Y rectangular system. The number of nucleoli per nuclear cross-section ( $n/N$ ) was calculated simply by dividing the number of nucleoli by the total number of nuclei. The location of the nucleolus was determined from the centres of gravity. The distance from the centre of gravity of the nucleus and the nucleolus was calculated with Pythagoras' theorem and called absolute nucleolar eccentricity (aNE). For a round or near-round nucleus the approximation of the centre of gravity for the centre of the cell is valid. However, this aNE has to be corrected for nuclear size to make it a reliable parameter for the position of

the nucleolus in the nucleus. This was done by dividing the aNE by the mean radius of the tumour cells, calculated from the mean nuclear area. The resulting parameter is a size-independent parameter varying from 0 (for a perfectly central nucleolus) to almost 1 (for a marginal one). We called it the relative nucleolar eccentricity (rNE). The method of assessing the nucleolar parameters has been described.<sup>16</sup>

The cytoplasmic parameters were also determined, but because it was often impossible to discern the contour, the assessment of these parameters was based on a smaller number of measurements. In most cases approximately 50 cells were traced. When the cytoplasmic contour was traced, the cytoplasm to nucleus ratio (C:N ratio) of that cell was calculated. In the cases of centroblastic/centrocytic lymphoma and polymorphic immunocytoma some blasts are present. To quantify these we designated the cell having a nuclear area  $> 35 \mu\text{m}^2$  as blasts and counted them. It is also known that immunoblasts can be found in centroblastic lymphoma. We attempted to quantify this by counting the cells with a nuclear area  $> 35 \mu\text{m}^2$ , one nucleolus and a rNE  $< 0.5$  in both centroblastic lymphoma and B immunoblastic lymphoma.

To check whether there were consistent differences between pathologists' interpretation of the selected areas and whether the method was reproducible, results of the photographs selected by one pathologist were compared with those selected by the other pathologist and the cells were remeasured by different persons.<sup>16,17</sup> The results showed a high degree of consistency, showing no systematical differences and a high reproducibility.

The differences between the types of lymphomas were statistically analysed using Wilcoxon's test.

## Results

The results of the morphometric measurements are summarised in Table 1. The statistically significant differences between the different lymphomas are summarised in Table 2.

### SMALL CELL LYMPHOMAS

The mean nuclear area showed uniformity with the exception of polymorphic immunocytoma and centroblastic/centrocytic lymphoma, both of which had slightly higher values due to the presence of blast cells. The SEM of the nuclear area, expressing the variation in cell size in the tumour, was of course also higher in these two lymphomas, but was also significantly lower for chronic lymphocytic leukaemia compared to all other small-cell lymphomas (Table 2). Differences in amount of cytoplasm and C:N ratio

Table 1 Mean and range of morphometrical data of nine types of B cell non-Hodgkin's lymphomas (a) small cell lymphomas (b) large cell and lymphoblastic lymphomas

Small cell lymphomas	Nuclear area ( $\mu\text{m}^2$ )		Cytoplasmic area ( $\mu\text{m}^2$ )		C:N ratio		NCI		% Blast cells <sup>†</sup>	Mean nuclear area without blasts
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM		
CLL (n = 5)	19.11 (16.7–21.5)	0.25* (0.2–0.3)	29.87 (27.7–31.7)	0.65 (0.5–0.8)	1.60* (1.5–1.8)	0.04 (0.03–0.05)	3.74* (3.7–3.8)	0.02 (0.01–0.03)		
LP (n = 5)	19.63 (14.6–22.1)	0.44* (0.4–0.5)	38.60 (26.7–44.8)	1.71 (1.3–2.3)	1.98* (1.9–2.2)	0.08 (0.04–0.14)	3.82* (3.7–3.9)	0.03 (0.01–0.03)		
PI (n = 5)	23.36* (19.5–25.5)	0.68* (0.5–0.9)	49.90 (41.2–64.0)	2.47 (1.8–2.9)	2.02* (1.8–2.2)	0.05 (0.04–0.07)	4.00* (3.9–4.1)	0.03 (0.02–0.03)	8.0 (5.0–16.9)	19.16 (16.23–20.5)
CC (n = 5)	18.44 (17.1–20.1)	0.40* (0.3–0.6)	28.06 (25.3–32.4)	1.00 (0.6–1.2)	1.57* (1.5–1.6)	0.04 (0.03–0.05)	4.18* (4.1–4.4)	0.04 (0.03–0.05)		
CB/CC (n = 5)	24.87* (2.39–26.5)	0.94* (0.7–1.2)	44.88 (42.0–49.6)	4.25 (3.2–7.3)	1.68* (1.6–1.8)	0.05 (0.03–0.07)	4.06* (4.0–4.3)	0.04 (0.03–0.05)	9.6 (8.0–12.4)	22.22 (21.7–23.1)
ILL (n = 5)	19.16 (16.6–21.1)	0.46* (0.4–0.6)	29.81 (28.2–31.4)	0.91 (0.7–1.3)	1.66* (1.5–1.8)	0.04 (0.04–0.05)	4.49* (4.3–4.7)	0.05 (0.04–0.07)		

Large cell and lymphoblastic lymphomas	Nuclear area ( $\mu\text{m}^2$ )		Cytoplasmic area ( $\mu\text{m}^2$ )		C:N ratio	
	Mean	SEM	Mean	SEM	Mean	SEM
CB (n = 9)	46.68 (30.1–57.1)	1.77* (0.8–2.5)	82.65 (51.6–102.3)	5.62* (2.3–9.5)	1.79* (1.6–2.0)	0.09 (0.06–0.13)
IB (n = 8)	37.61 (27.1–47.2)	1.58* (1.4–2.0)	72.33 (54.6–107.6)	4.83* (3.1–7.1)	2.04* (1.8–2.3)	0.09 (0.05–0.14)
LB (n = 5)	39.64 (27.9–54.0)	0.91* (0.7–1.1)	66.66 (45.0–88.7)	1.78* (1.5–2.1)	1.74 (1.6–1.9)	0.04 (0.02–0.06)

Large cell and lymphoblastic lymphomas	NCI		% Immunoblasts <sup>‡</sup>	Mean nucleolar area	rNE	n/N
	Mean	SEM				
CB (n = 9)	4.07 (3.9–4.3)	0.04 (0.02–0.05)	18.5* (10.1–28.3)	2.66 (1.7–4.2)	0.59* (0.5–0.7)	1.30* (0.9–1.6)
IB (n = 8)	4.05 (3.8–4.3)	0.04* (0.02–0.06)	43.4* (31.2–67.0)	3.17 (2.4–4.2)	0.42* (0.3–0.5)	0.91* (0.8–1.0)
LB (n = 5)	3.98 (3.8–4.1)	0.02 (0.01–0.03)	—	—	—	—

CLL = chronic lymphocytic leukaemia.

PI = polymorphic immunocytoma.

CC = centrocytic lymphoma.

CB = centroblastic lymphoma.

LB = lymphoblastic lymphoma, Burkitt type.

LP = lymphoplasmacytoid immunocytoma.

CB/CC = centroblastic/centrocytic lymphoma.

ILL = intermediate lymphocytic lymphoma.

IB = B immunoblastic lymphoma.

C:N ratio = cytoplasm to nucleus ratio.

NCI = nuclear contour index.

SEM = standard error of mean.

rNE = relative nucleolar eccentricity.

n/N = mean number of nucleoli per nuclear cross-section.

( ) = range.

\* Marks the values that showed up the important and significant differences between the lymphomas (see Table 2).

<sup>†</sup>Blast cells = cells with nuclear cross-sectional area  $>35 \mu\text{m}^2$ .

<sup>‡</sup>Immunoblast defined as a cell with a nuclear cross-sectional area  $>35 \mu\text{m}^2$ , and one nucleolus with a rNE  $<0.5$ .

were subtle. Polymorphic immunocytoma and centroblastic/centrocytic lymphoma had the largest cytoplasmic area values, significantly larger than in other small cell lymphomas ( $0.008 < p < 0.01$ ), except for lymphoplasmacytoid immunocytoma, which did not have a significantly smaller mean cytoplasmic area, but had a significantly lower SEM ( $p = 0.02$ ). Lymphoplasmacytoid immunocytoma and polymorphic immunocytoma had the highest C:N ratio values, but this differed significantly only from centrocytic lymphoma and intermediate lymphocytic lymphoma ( $p = 0.01$ ). The mean NCI was lowest for chronic lymphocytic leukaemia,

significantly lower than for lymphoplasmacytoid immunocytoma ( $p = 0.008$ ). Centroblastic/centrocytic lymphoma and polymorphic immunocytoma had significantly higher values than lymphoplasmacytoid immunocytoma ( $p = 0.01$ ), centrocytic lymphoma had significantly higher mean NCI than centroblastic/centrocytic lymphomas and polymorphic immunocytoma ( $p = 0.02$ ), while intermediate lymphocytic lymphoma had the most irregular nuclei as expressed by the mean NCI, significantly higher than for centrocytic lymphoma ( $p = 0.03$ ). The percentage of blasts (defined as a cell with a nuclear area  $>35 \mu\text{m}^2$ ) was determined in the

Table 2 Comparison of lymphomas on the basis of morphometrical data and statistical analysis

Lymphoma	Compared with	Statistically significant differences	Histological implications
CLL	LP	SE of nuclear area (p = 0.008). Mean NCI (p = 0.01)	CLL cells have more monomorphic and round nuclei.
CLL	PI	Mean nuclear area (p = 0.04). SE of nuclear area (p = 0.008). Mean cytoplasmic area (p = 0.008). Mean NCI (p = 0.008)	CLL cells have more monomorphic and round nuclei, less cytoplasm. There is admixture with blast cells in PI.
CLL	CC	SE of nuclear area (p = 0.008). Mean NCI (p = 0.008)	CLL cells have more monomorphic and round nuclei.
CLL	CB/CC	Mean nuclear area (p = 0.008). SE of nuclear area (p = 0.008). Mean cytoplasmic area (p = 0.008). Mean NCI (p = 0.008)	CLL cells have more monomorphic and round nuclei, less cytoplasm. There is admixture with blast cells.
CLL	ILL	SE of nuclear area (p = 0.008). Mean NCI (p = 0.008)	CLL cells have more round nuclei, ILL cells have markedly indented nuclei.
LP	CC	SE of cytoplasmic area (p = 0.02). Mean C:N ratio (p = 0.01). Mean NCI (p = 0.01). SE of NCI (p = 0.02)	Centrocytes have more cleaved nuclei, and less cytoplasm.
LP	PI	SE of nuclear area (p = 0.01). SE of cytoplasmic area (p = 0.02). Mean NCI (p = 0.01)	LP cells show less variation in nuclear area and shape, and cytoplasmic area. Variation in nuclear size points to presence of blasts in PI.
LP	CB/CC	Mean nuclear area (p = 0.01). SE of nuclear area (p = 0.01). SE of cytoplasmic area (p = 0.01). Mean C:N ratio (p = 0.01). Mean NCI (p = 0.01). SE of NCI (p = 0.02)	LP cells are more round and have more cytoplasm. Variation in nuclear area points to blast cells present in CB/CC.
LP	ILL	SE of cytoplasmic area (p = 0.02). Mean C:N ratio (p = 0.01). Mean NCI (p = 0.01). SE of NCI (p = 0.01)	ILL cells have more irregular nuclei and less cytoplasm.
PI	CC	Mean nuclear area (p = 0.02). SE of nuclear area (p = 0.04). Mean cytoplasmic area (p = 0.01). SE of cytoplasmic area (p = 0.01). Mean C:N ratio (p = 0.01). SE of C:N ratio (p = 0.04). Mean NCI (p = 0.02)	Blasts present in PI. Centrocytes have more irregular nuclei and less cytoplasm.
PI	CB/CC	Mean C:N ratio (p = 0.01). SE of NCI (p = 0.03)	Cells in PI show less variance in nuclear shape, have more cytoplasm.
PI	ILL	Mean nuclear area (p = 0.01). SE of nuclear area (p = 0.04). Mean cytoplasmic area (p = 0.01). SE of cytoplasmic area (p = 0.01). Mean C:N ratio (p = 0.01). SE of C:N ratio (p = 0.04). Mean NCI (p = 0.01). SE of NCI (p = 0.01)	Blasts present in PI. ILL cells have more irregular nuclei, and less cytoplasm.
CC	CB/CC	Mean nuclear area (p = 0.01). SE of nuclear area (p = 0.01). Mean cytoplasmic area (p = 0.01). SE of cytoplasmic area (p = 0.01). Mean C:N ratio (p = 0.03)	Cells in CB/CC show more variance in nuclear area: blasts are present in CB/CC. Cells in CB/CC have more cytoplasm.
CC	ILL	Mean C:N ratio (p = 0.04). Mean NCI (p = 0.03)	C:N ratio lower for centrocytes. ILL cells have more irregular nuclei.
CB/CC	ILL	Mean nuclear area (p = 0.01). SE of nuclear area (p = 0.01). Mean cytoplasmic area (p = 0.01). SE of cytoplasmic area (p = 0.01). Mean NCI (p = 0.02). SE of NCI (p = 0.04)	CB/CC contains blast cells, cells in ILL have more irregular nuclei, smaller cytoplasmic area.
CB	IB	Mean C:N ratio (p = 0.02). rNE (p < 0.00005). n/N (p < 0.0005)	Centroblasts have a lower C:N ratio, a higher number of nucleoli per nucleus, and more peripherally located nucleoli.
CB	LB	SE of nuclear area (p = 0.02). SE of cytoplasmic area (p = 0.02). SE of NCI (p < 0.05)	LB shows a more uniform histological picture.
IB	LB	SE of nuclear area (p = 0.006). SE of cytoplasmic area (p = 0.006). Mean C:N ratio (p = 0.01). SE of C:N ratio (p = 0.01). SE of NCI (p = 0.03)	LB shows a more uniform histological picture. Lymphoblasts have less cytoplasm.

CLL = chronic lymphocytic leukaemia.

LP = lymphoplasmacytoid immunocytoma.

PI = polymorphic immunocytoma.

CC = centrocytic lymphoma.

CB/CC = centroblastic/centrocytic lymphoma.

ILL = intermediate lymphocytic lymphoma.

CB = centroblastic lymphoma.

IB = B immunoblastic lymphoma.

LB = lymphoblastic lymphoma, Burkitt type.

SE = standard error of mean.

NCI = nuclear contour index.

C:N ratio = cytoplasm to nuclear ratio.

cases of centroblastic/centrocytic lymphoma and polymorphic immunocytoma. Though the percentages varied somewhat, most remained below 10%, the highest being 16.9%. With the eight parameters each lymphoma could be differentiated from the others. The various differences, their significances and the histological implications of this are presented in Table 2.

#### LARGE CELL LYMPHOMAS

No significant differences were found between

centroblastic lymphoma and B immunoblastic lymphoma with regards to their nuclear and cytoplasmic area and NCI. The striking variation in nuclear size, as well as the high SEM reflected the varying admixture of small cells with the blastic cells. A small but significant difference was found for the C:N ratio, with B immunoblastic lymphoma having higher values. The mean rNE and mean n/N were clearly different for these two lymphomas. The centroblastic lymphomas had mean rNE values > 0.5 whereas all B immunoblastic lymphomas had values








	<u>Cell type</u>	<u>Markers</u>	<u>Morphometric Characteristics</u>
	B-lymphocyte of CLL	sIg + cIg - C <sub>3</sub> +/+	NA : 15 - 20µm <sup>2</sup> NCI: ±3.7 C/N: 1.5 - 1.6
	Lymphoplasmacytoid cell	sIg + cIg + C <sub>3</sub> -/±	NA : 15 - 20µm <sup>2</sup> NCI: ±3.8 C/N: 1.8 - 2.0
	Centrocyte	sIg + cIg - C <sub>3</sub> ++	NA : 15 - 20µm <sup>2</sup> NCI: 4.0 - 4.2 C/N: 1.5 - 1.6
	"Intermediate" Lymphocyte	sIg + cIg - C <sub>3</sub> +	NA : 15 - 20µm <sup>2</sup> NCI: 4.2 - 4.7 C/N: 1.5 - 1.8
	B-Immunoblast	sIg + cIg + C <sub>3</sub> -/+	NA : 30 - 50µm <sup>2</sup> NCI: 3.8 - 4.2 C/N: 1.8 - 2.3
	Centroblast	sIg + cIg -/+ C <sub>3</sub> +/++	NA : 30 - 60µm <sup>2</sup> NCI: 3.9 - 4.3 C/N 1.6 - 2.0
	B-lymphoblast of Burkitt type	sIg + cIg - C <sub>3</sub> ±	NA : 25 - 45µm <sup>2</sup> NCI: 3.9 - 4.1 C/N: 1.6 - 1.9

Fig. 1 Morphometric and marker characteristics of the measured mean cell type in B cell non-Hodgkin's lymphomas.

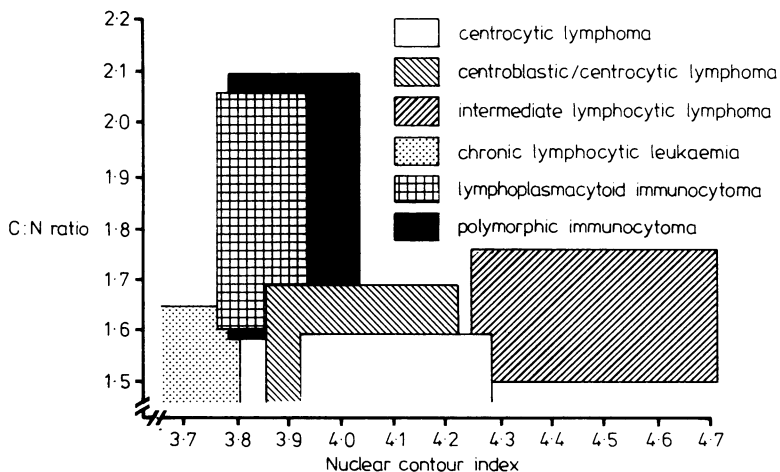


Fig. 2 Schematic representation of differences between types of small cell lymphomas

< 0.5 ( $p < 0.0005$ ). The mean  $n/N$  was less discriminating but still useful, centroblastic lymphoma having higher values—that is, more nucleoli, than in B immunoblastic lymphoma. The nucleolar area did not discriminate. The number of immunoblasts, as defined by the rigid criteria mentioned before, showed predictable differences, but up to 28.3% of these cells were found in one case of centroblastic lymphoma. Comparison between the large cell lymphomas and lymphoblastic lymphomas can be found in Table 2.

#### Lymphoblastic lymphomas (Burkitt type)

Of the five cases studied three were in the range of the large cell lymphomas, two were intermediate-sized. They had moderate amounts of cytoplasm and near-round nuclei. They differed from the large cell lymphomas in the SEM of all studied parameters—that is, they showed less variation than the large cell lymphomas.

The morphometric characteristics are represented in Fig. 1.

#### Discussion

The results of this study show that morphometry of plastic-embedded sections is valuable for the differentiation not only of small cell and large cell lymphomas but also of subtypes of these lymphomas. However, our measurements also show how subtle the differences are. Use of plastic-embedded sections is therefore necessary as recommended by Abbott *et al.*<sup>19</sup> and van der Valk *et al.*<sup>16</sup> In our experience the measurements on paraplast-embedded sections show a greater variability, due to shrinkage and inadequate stretching of the slides. This is undesirable in a group where the differences are so small.

The small cell lymphomas can be separated into types as shown in Table 2. A graphic representation of the differences between small cell lymphomas based on two morphometrical parameters—that is, NCI and C:N ratio, is shown in Fig. 2. It is thus possible to differentiate between chronic lymphocytic leukaemia and lymphoplasmacytoid immunocytoma, as chronic lymphocytic leukaemia shows a more monomorphic nuclear picture than lymphoplasmacytoid immunocytoma. Likewise the NCI allows a distinction between chronic lymphocytic leukaemia and lymphoplasmacytoid immunocytoma on the one hand and centrocytic lymphoma and intermediate lymphocytic lymphoma on the other. Intermediate lymphocytic lymphoma is a relatively recently described lymphoma<sup>5, 20-23</sup> and appears to be a clinicopathological entity.<sup>5</sup> On morphometry, it is distinguishable from the other small cell lymphomas,

as it has very high NCI values, even higher than centrocytic lymphoma. On nuclear size, polymorphic immunocytoma and centroblastic/centrocytic lymphoma were distinguishable, due to the presence of blasts. The variability in the number of blasts created some heterogeneity. This is reflected in the mean and SEM of their nuclear area; the higher these parameters, the higher the percentage of blasts. In the present lymphoma concepts<sup>24</sup> a relation is assumed between the number of blast cells in a lymphoma and the clinical behaviour of the tumour. Therefore the assessment of the percentage of the blasts is important. Differentiation between centroblastic/centrocytic and centroblastic lymphoma is also made easier. Lennert<sup>1</sup> calls a lymphoma centroblastic/centrocytic if less than 25% of the cells are centroblasts. With morphometry this percentage can be confirmed and a relation of these results to the clinical behaviour can be evaluated. Morphometry of blast cells allows the distinction to be made between polymorphic immunocytoma and B immunoblastic lymphoma; the percentage of blast cells can then be related to clinical behaviour.

Thus our study showed that the small cell lymphomas can be differentiated by the mean and SEM of the nuclear area (variation in nuclear size), the C:N ratio (relative amount of cytoplasm) and NCI (nuclear irregularity). Sotto *et al.*<sup>25</sup> also found in a cytological study of non-Hodgkin's lymphomas, that cell size and the ratio of cytoplasm to nucleus were important criteria. The large cell lymphomas CB and IB were distinguishable on their nucleolar parameters and C:N ratio. These differences have been mentioned,<sup>1, 16, 26, 27</sup> but it is also stressed that these differences overlap. Our findings underline this. The two groups seem to join or, as the  $n/N$  shows, overlap. As is suggested<sup>1, 25</sup> these neoplasms are probably closely related. Nevertheless, there are differences in rNE and C:N ratio allowing these two lymphomas to be distinguished from one another. Centroblastic lymphoma has a larger number of, and more peripherally located nucleoli, and relatively less cytoplasm than B-immunoblastic. In a previous study<sup>16</sup> the difference in C:N ratio was not significant, but here one case was added, making the difference significant ( $p < 0.05$ ). As this method allows the distinction of immunoblasts as they are defined in the literature<sup>1</sup>—that is, a cell with a large nucleus with a large, central nucleolus, admixture of immunoblasts in centroblastic lymphoma can be defined. This may be related to clinical parameters. As B-immunoblastic lymphomas behave more aggressively than centroblastic lymphomas,<sup>3</sup> it is of interest to find out if an increasing number of immunoblasts has a negative influence on the clinical behaviour. The possible correlation between the number of

immunoblasts and clinical behaviour is currently under study.

The lymphoblastic lymphomas of this study fall in the same size range as the B-immunoblastic lymphomas and centroblastic lymphomas. Lymphoblastic lymphoma (Burkitt type) is generally considered intermediate-sized,<sup>1</sup> but Lennert<sup>1</sup> already mentioned the existence of a lymphoblastic lymphoma (Burkitt type) as large as a centroblastic lymphoma. Indeed, the lymphoblastic lymphoma of this study resembles the centroblastic lymphoma closely. However, the SEM of the nuclear area of lymphoblastic lymphoma is significantly lower, indicating a more monomorphic cellular composition. As Nathwani *et al*<sup>28</sup> report on some cases of polymorphic lymphoblastic lymphoma, distinguishing between lymphoblastic lymphoma and centroblastic lymphoma can be very difficult.

In conclusion our study shows that significant morphometric differences could be found between 52 B cell non-Hodgkin's lymphomas, grouped according to the Kiel classification. Therefore, these findings endorse the Kiel classification. They also support the existence of the group of intermediate lymphocytic lymphoma. Together with the immunological and cytochemical techniques and electron microscopy, morphometry on plastic-embedded sections is a useful technique for the differentiation of non-Hodgkin's lymphomas of B cell origin. Our results also suggest that in overlap areas, such as centroblastic/centrocytic to centroblastic lymphomas or polymorphic immunocytomas to B-immunoblastic lymphomas, morphometry can be of great help in defining these lymphomas more precisely.

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## References

- Lennert K. *Malignant lymphomas other than Hodgkin's disease*. Berlin: Springer-Verlag, 1978.
- Nathwani BN, Kim H, Rappaport H, Solomon J, Fox M. Non-Hodgkin's lymphomas. A clinicopathological study comparing two classifications. *Cancer* 1978;41:303-25.
- Musshoff K, von Stotzingen W, Schmidt-Volmer H, Umbach H. Investigation results of a comparison made between the Kiel and Rappaport classification of the non-Hodgkin's lymphomas, together with clinical data. *J Cancer Res Clin Oncol* 1981;100:167-204.
- Gerard-Marchant R, Hamlin I, Lennert K, Rilke F, Stansfeld AG, Van Unnik JAM. Classification of non-Hodgkin's lymphomas. *Lancet* 1974;iii:406.
- Weisenburger DD, Nathwani BN, Diamond LW, Winberg CD, Rappaport H. Malignant lymphoma, intermediate lymphocytic type. A clinicopathological study of 42 cases. *Cancer* 1981;48:1415-25.
- McDowell EM, Trump BF. Histologic fixation suitable for diagnostic light and electronmicroscopy. *Arch Pathol Lab Med* 1976;100:405.
- Bosman FT, Kreuning J, Van der Wal AM, Kuiper G, Lindeman J. The influence of fixation on immunoperoxidase staining of plasma cells in paraffin sections of intestinal biopsy specimens. *J Histochem* 1977;53:57-62.
- Te Velde J, Burkhardt R, Kleiverda K, Leenheers-Binnendijk L, Sommerfield W. Methylmethacrylate as an embedding medium in histopathology. *Histopathology* 1977;1:319-30.
- Van der Valk P, Te Velde J, Jansen J, *et al*. Malignant lymphoma of true histiocytic origin: histiocytic sarcoma. A morphological, ultrastructural, immunological, cytochemical and clinical study of 10 cases. *Virchows Arch [Pathol Anat]* 1981;391:249-65.
- Sternberger LA. *Immunocytochemistry*. London: Prentice-Hall, 1977:53-4.
- Meijer CJLM, Lindeman J. A modified method for tissue localisation of cells bearing a complement receptor. *J Immunol Methods* 1975;9:59-65.
- Meijer CJLM, Van de Putte LBA, Eulderink F, Kleinjan R, Lafeber C, Bots GThAM. Characteristics of mononuclear cell populations in chronically inflamed synovial membranes. *J Pathol* 1977;121:1-8.
- Wachstein M, Meisel E. Histochemistry of hepatic phosphatases at a physiological pH. With a special reference to the demonstration of bile canaliculi. *Am J Clin Pathol* 1957;27:13-23.
- Barka T, Anderson PJ. *Histochemistry. Theory, practice and bibliography*. 2nd ed. New York: Harper Row, 1965.
- Gomori G. The study of enzymes in tissue sections. *Am J Clin Pathol* 1964;16:347-52.
- Van der Valk P, Hermans J, Brand R, Cornelisse CJ, Spaander PJ, Meijer CJLM. Morphometric characterisation of diffuse large-cell (histiocytic) lymphomas. *Am J Pathol* 1982;107:327-35.
- Van der Loo EM, Cornelisse CJ, Van Vloten WA, Van der Velde EA, Scheffer E, Meijer CJLM. Diagnostic morphometry of isolated lymph node cells from patients with mycosis fungoides and the Sézary syndrome. *Virchows Arch [Cell Pathol]* 1980;33:107-16.
- Meijer CJLM, Van der Loo EM, Van Vloten WA, Van der Velde EA, Scheffer E, Cornelisse CJ. Early diagnosis of mycosis fungoides and Sézary's syndrome by morphometric analysis of lymphoid cells in the skin. *Cancer* 1980;45:2864-71.
- Abbott CR, Blewitt RW, Bird CC. Quantitative analysis of non-Hodgkin's lymphoma. *J Clin Pathol* 1982;35:135-8.
- Braylan RC, Jaffé ES, Berard CW. Malignant lymphoma: current classification and new observations. *Pathol Annu* 1975;213-70.
- Jaffé ES, Braylan RC, Nanba K, Frank MM, Berard CW. Functional markers: a new perspective on malignant lymphomas. *Cancer Treat Rep* 1977;61:953-62.
- Nanba K, Jaffé ES, Braylan RC, Soban EJ, Berard CW. Alkaline phosphatase-positive malignant lymphoma. A subtype of B-cell lymphomas. *Am J Clin Pathol* 1977;68:535-42.
- Mann RB, Jaffé ES, Berard CW. Malignant lymphomas—a conceptual understanding of morphologic diversity. *Am J Pathol* 1979;94:104-91.
- The non-Hodgkin's lymphoma pathologic classification project: National Cancer Institute sponsored study of non-Hodgkin's lymphomas summary and description of a working formulation for clinical usage. *Cancer* 1982;49:2112-35.
- Sotto JJ, Caillot P, Sotto MF, *et al*. Non-Hodgkin's malignant lymphomas. A cytological classification based on statistical analysis and correlated with prognosis. *Anal Quant Cytol* 1982;4:6-25.
- Lukes RJ, Collins RD. New approaches to the classification of the lymphomata. *Br J Cancer* 1975;31, suppl II:1-28.
- Lukes RJ, Parker JW, Talor CR, Tinde BH, Cramer AD, Lincoln TL. Immunologic approach to non-Hodgkin's lymphomas and



related leukemias. Analysis of the results of multiparameter studies of 425 cases. *Semin Hematol* 1978;15:322-51.

<sup>28</sup> Nathwani BN, Diamond LW, Winberg CD, *et al.* Lymphoblastic lymphoma. A clinicopathologic study of 95 patients. *Cancer* 1981;48:2347-57.

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