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The morphology of CLL revisited: the clinical significance of prolymphocytes and correlations with prognostic/molecular markers in the LRF CLL4 trial

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Summary

Historically, an increase in the percentage and number of circulating prolymphocytes in chronic lymphocytic leukaemia (CLL) has been associated with strong expression of surface immunoglobulin, trisomy 12 and a poor outcome. This study re-examines the biological and clinical significance of increased peripheral blood prolymphocytes in 508 patients at entry into the randomized UK Leukaemia Research Fund CLL4 trial. It also investigates the associations between increased prolymphocytes and a comprehensive array of biomarkers. 270 patients (53%) had <5% prolymphocytes, 167 (33%) had 5–9%, 60 (12%) had 10–14% and 11 (2%) had ≥15% prolymphocytes. We show that a higher proportion of prolymphocytes (≥10%) was independently associated with NOTCH1 mutations (P = 0.006), absence of 13q deletion (P = 0.001), high CD38 expression (P = 0.02) and unmutated IGHV genes (P = 0.01). Deaths due to Richter syndrome were significantly more common amongst patients who had ≥10% vs <10% prolymphocytes (13% vs 2%) respectively (P < 0.0001). ≥10% prolymphocytes was also associated with a shorter progression-free survival (Hazard ratio [HR] 1.50 [95% confidence interval [CI]: 1.16–1.93], P = 0.002) and overall survival (HR 1.99 [95% CI: 1.53–2.59], P < 0.0001). Our data support the routine examination of blood films in CLL and suggest that a finding of an increased proportion of prolymphocytes may be a trigger for further evaluation of clinical and laboratory features of progressive disease.

Keywords: Chronic lymphocytic leukaemia, prolymphocytes, prognostic markers, molecular markers, morphology.

Although peripheral blood lymphocytes in chronic lymphocytic leukaemia (CLL) are typically small with clumped chromatin and scanty cytoplasm, it has long been recognized that a subset of patients present with, or acquire, an increased percentage of lymphocytes that are larger with more abundant cytoplasm, nuclear irregularities, lymphoplasmacytoid features and/or one or more prominent nucleoli.

Early studies evaluating the clinical significance of lymphocyte morphology were confounded by difficulties in distinguishing CLL from other chronic lymphoproliferative disorders and gave disparate results (Peterson et al, 1975; Dubner et al, 1978). However, following the initial observation that increasing refractoriness to treatment may be accompanied by the appearance of prolymphocytes in the blood (Enno et al, 1979), a detailed analysis of 300 cases with either CLL (n = 258) or B-cell prolymphocytic leukaemia (PLL) (n = 42) identified ≥55% circulating prolymphocytes as a defining diagnostic criterion for PLL, and CLL cases with 11–55% circulating prolymphocytes (CLL/PLL) as having clinical features intermediate between typical CLL and prolymphocytic leukaemia; namely a higher incidence of splenomegaly and higher intensity of surface immunoglobulin expression (SmIg) than in typical CLL (Melo et al, 1986). Within the CLL/PLL group, those patients with an absolute prolymphocyte count of ≥15 × 10⁹/l were shown to have a shorter overall survival (OS) than those with a lower absolute prolymphocyte count (Melo et al, 1987).

Although the adverse prognostic significance of increased prolymphocytes was subsequently confirmed in other studies (Scott et al, 1987; Vallespi et al, 1991; Criel et al, 1997;
Oscier et al., 1997), the role of morphological examination of blood films in CLL as a guide to prognosis has diminished with the discovery of multiple newer biomarkers.

The Leukaemia Research Fund (LRF) CLL4 trial randomized previously untreated patients to either chlorambucil or fludarabine, alone or in combination with cyclophosphamide (FC) (Catovsky et al., 2007). At randomization, differential white blood cell counts were performed and this has provided an opportunity to re-evaluate both the prognostic significance of nucleolated cells (prolymphocytes and immunoblasts) regarding progression-free survival (PFS) and OS as well as their correlation with established and recently identified prognostic markers. We found that increased prolymphocytes were associated with markers of poor prognosis and predicted a shorter PFS and OS.

**Patients and methods**

In the LRF CLL4 trial 777 patients were randomized between February 1999 and October 2004 to receive chlorambucil, fludarabine or FC. The patients were previously untreated, 25% having Binet stage A-progressive disease, 45% stage B and 30% stage C. The male:female ratio was 3:1 and the median age was 65 years (range 35–86 years). Clinical follow-up was to 31 October 2010, and follow-up for OS for UK patients was to January 2015 (median 11.8 years; range 10.2–15.9 years). In the UK, the deaths of CLL trial patients are flagged and reported to the Clinical Trial Service Unit at Oxford. For 44 surviving patients resident outside the UK, for whom this information was not available, OS was censored at 31 October 2010.

Differential white blood cell counts were performed on slides stained with May Grunwald Giemsa from blood samples taken at trial entry in 508 patients. A total of 200 cells were scored in patients with lymphocyte counts below 25 × 10⁹/l and 300 cells in the remaining cases. Lymphoid cells were classified according to the criteria of Melo et al. (1986). Specifically, prolymphocytes were defined as large cells (>2 erythrocytes) with clumped chromatin, a large prominent vesicular nucleolus and usually abundant cytoplasm. Immunoblasts were larger (>3 erythrocytes) with finely dispersed chromatin, large and usually >1 nucleoli, and deeply basophilic cytoplasm (Fig. 1). When present, immunoblasts were rare and were included within the prolymphocyte count.

The choice of cut-off to define an increase in prolymphocytes was based on previous studies of the clinical and biological features of CLL cases with increased prolymphocytes. To determine associations with other variables a 10% cut-off was used, as this defines the distinction between CLL and CLL/PL. To evaluate the impact on clinical outcome, both a 10% cut-off and an absolute prolymphocyte count of ≥15 × 10⁹/l were used, as the latter was found to be the best discriminator of outcome within CLL/PL (Melo et al., 1987). The clinical value of a 10% cut-off was also confirmed in our analysis of PFS and OS (see results).

Immunophenotypic analysis was performed centrally by flow cytometry using a panel of monoclonal antibodies: CD5, CD19, CD20, CD23, CD79b, FMC7, and surface light chain immunoglobulins, enabling a CLL score to be derived (Moreau et al., 1997). All cases with a low score were also reviewed. Those cases with a phenotype lacking expression of CD23 or CD5, probably reflecting prolonged transit in the postal system or an alternative diagnosis, were only included if lymphocyte morphology was typical of CLL and fluorescent in situ hybridization (FISH) for t(11;14) was negative, particularly in CD23 negative cases. Data on the following

![Morphological appearances of chronic lymphocytic leukaemia (CLL) and CLL with >10% circulating prolymphocytes (CLL/PL).](image)

(A) Typical CLL. The majority of cells are small with clumped chromatin. (B) Typical CLL/PL. There is a mixture of prolymphocytes and typical CLL lymphocytes. (C) Typical CLL/PL showing small lymphocytes, prolymphocytes and an immunoblast. (D) Typical B-cell prolymphocytic leukaemia (B-PLL). The majority of cells have condensed non-clumped chromatin and a single vesicular nucleolus. This panel is shown here for comparative purposes only, to illustrate the similar morphology of the prolymphocytes in B-PLL to those seen in panels A and C. The majority of cells in B-PLL are prolymphocytes and no small lymphocytes are seen. The usual “CLL score” is 0–1. B-PLL is a distinct disorder and does not arise from a pre-existing CLL. Magnification: panels A, B and C ×100; panel D ×60.
markers were available: FISH to detect 11q, 13q, 17p deletions and trisomy 12, IGHV mutation status, CD38, ZAP70 and CLLU1 expression, serum beta-2 microglobulin (B2M), TP53, SF3B1 and NOTCH1 mutations and telomere length as reported elsewhere, together with a full description of the cut-offs used to define positivity (Oscier et al, 2010, 2013; Gonzalez et al, 2011, 2013; Strefford et al, 2015).

The LRF CLL4 trial was registered as an International Standard Randomized Trial, number ISRCTN58585610 and was approved by a UK multicentre research ethics committee. The trial followed the UK Medical Research Council guidelines for good clinical practice. All patients provided written informed consent. All authors had access to the primary clinical trial data. The main trial endpoints have been previously reported (Catovsky et al, 2007).

Survival was estimated by the Kaplan-Meier method. OS was calculated from randomization to death from any cause. DFS was estimated from the time of randomization to relapse needing further treatment, progression or death from any cause. For non-responders (NR) and those with progressive disease (PD), date of progression was when NR/PD was recorded. Multivariate analyses were performed by means of stepwise generalized linear modelling and the Cox proportional hazards model. Values of \( P \leq 0.05 \) (two sided) were considered significant. Analyses were performed using the STATISTICA software from StatSoft, a subsidiary of Dell, Inc. (Tulsa, OK, USA).

Results

Of 508 assessable patients, 270 (53%) had <5% prolymphocytes, 167 (33%) had 5–9%, 60 (12%) had 10–14% and 11 (2%) had ≥15% prolymphocytes. Among the 504 patients in whom an absolute lymphocyte count was available, the absolute prolymphocyte count was \(<15 \times 10^9/l\) in 442 (88%) and \(\geq 15 \times 10^9/l\) in 62 (12%) patients. These groups were equally distributed between the three trial arms. There was a tendency for younger patients, those with stage B disease, a low white blood count, 11q deletion or high CLLU1 expression to be moderately under-represented amongst the 508 trial patients who had prolymphocyte data, but otherwise the clinical and molecular characteristics of this subset were the same as those of the 269 patients without available prolymphocyte data (Table SI). Thus the subset with prolymphocyte data was broadly representative of the trial as a whole.

Association of increased prolymphocytes with immunophenotype

Eighty-eight per cent of patients had a CLL score of 4 or 5 and 7% had a score of 3. There was no correlation between the CLL score and the percentage of prolymphocytes. However there was a significant association between ≥10% prolymphocytes and strong expression of SmIg (\( P < 0.0001 \)).

<table>
<thead>
<tr>
<th>CLL score component</th>
<th>≥10% prolymphocytes (n)</th>
<th>≥15% prolymphocytes (n)</th>
<th>P-value (≥10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMC7 negative</td>
<td>403 59 (15)</td>
<td>7 (2)</td>
<td>NS</td>
</tr>
<tr>
<td>FMC7 positive</td>
<td>91 10 (11)</td>
<td>3 (3)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>SmIg weak</td>
<td>371 37 (10)</td>
<td>5 (1)</td>
<td></td>
</tr>
<tr>
<td>SmIg strong</td>
<td>123 32 (26)</td>
<td>5 (4)</td>
<td></td>
</tr>
<tr>
<td>CD23 positive</td>
<td>462 67 (14-5)</td>
<td>10 (2)</td>
<td>NS</td>
</tr>
<tr>
<td>CD23 negative</td>
<td>32 2 (6)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>CD379b weak</td>
<td>437 59 (13-5)</td>
<td>9 (2)</td>
<td>NS</td>
</tr>
<tr>
<td>CD379b strong</td>
<td>57 10 (18)</td>
<td>1 (2)</td>
<td></td>
</tr>
<tr>
<td>CD5 positive</td>
<td>490 69 (14)</td>
<td>10 (2)</td>
<td>NS</td>
</tr>
<tr>
<td>CD5 negative</td>
<td>4 0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

OS, overall survival.

Association of increased prolymphocytes with other clinical and laboratory features

Table II summarizes the degree of association between ≥10% prolymphocytes and other clinical and laboratory features. No associations were found with treatment arm, age, Binet stage, the presence of lymphadenopathy or splenomegaly, TP53 deletion or mutation, deletion of 11q, SF3B1 mutation or ZAP70 expression. Because the variables were available in different sub-sets of patients, multivariate analysis of the significant variables was performed in consecutive stages, beginning with only the variables that were available from the majority (\( n = 460 \)) of the 508 patients with prolymphocyte data. Absolute prolymphocyte count was not included because of its close relationship, by definition, to % prolymphocytes. Gender, white blood count, 13q deletion and trisomy 12 were each independently significant in this first stage. We then modelled the other significant variables (Table II) one at a time together with the above four variables. Those which retained independent significance were taken forward to a final model (\( n = 256 \)) which included gender, white blood cell count, IGHV mutation status, 13q deletion, trisomy 12, NOTCH1 mutation and CD38 expression. Four variables were independently associated with percentage of prolymphocytes (Table III).

Association of increased prolymphocytes with outcome

There was no significant difference in first-line overall response rate (74% vs 80%, \( P = 0.3 \)) or first line CR rate...
(17% vs 18%, \( P = 0.9 \)) for patients with \( \geq 10\% \) vs \(<10\% \) prolymphocytes respectively.

PFS following initial treatment was worse for patients with \( \geq 10\% \) prolymphocytes (Hazard ratio [HR] 1.50 [95% confidence interval (CI): 1.16–1.93], \( P = 0.002 \)) (Fig 2A) and for those with \( \geq 15 \times 10^9/l \) prolymphocytes (HR 1.45 [95% CI: 1.11–1.91], \( P = 0.007 \)) (Fig 2B). The 5-year PFS was 4% (95% CI: 0–9%) for patients with \( \geq 10\% \) prolymphocytes vs...
17% (14–21%) for those with <10% prolymphocytes. OS was also worse for patients with ≥10% prolymphocytes (HR 1.99 [95%CI: 1.53–2.59], P < 0.0001) (Fig 2C), and for those with ≥15 × 10⁹/l prolymphocytes (HR 1.53 [95% CI: 1.15 – 2.04], P = 0.004) (Fig 2D). OS at 10 years was 3% (0–7%) vs 30% (26–35%) for patients with ≥10% vs <10% prolymphocytes respectively. The adverse prognostic significance of ≥10% prolymphocytes for both PFS and OS was evident in each arm of the trial (Figure S1).

Given that the choice of a 10% cut-off to define increased prolymphocytes was based on historical data, we then compared the PFS and OS for each of four groups defined by % prolymphocytes ranging from <5% to ≥15% (Fig 3). This shows a significant reduction in both PFS and OS compared to cases with <5% prolymphocytes, irrespective of whether the cut-off was 5% (PFS P = 0.0004, OS P = 0.0006) or 10% (PFS P = 0.006, OS P < 0.0001), but provides no support for a cut-off of >15% (PFS P = 0.3, OS P = 0.07) which represents only 2% of cases.

Only 8/71 (11%) patients with ≥10% prolymphocytes received no further treatment during the clinical follow-up period of the trial (median 7 years, range 6–12 years) compared with 146/437 (33%) of those with <10% prolymphocytes (P = 0.0002). Two or more further lines of treatment were given to 37/71 (52%) patients with ≥10% prolymphocytes, but to only 134/437 (31%) patients with <10% prolymphocytes (P = 0.0004).

To date 17/508 patients (3%) have died as a result of Richter syndrome. Such deaths were significantly more common amongst patients who had ≥10% prolymphocytes at randomization (9/71, 13%) vs those with <10% prolymphocytes (8/437, 2%; P < 0.0001).

As with the analysis of factors associated with % prolymphocytes, the multivariate analysis to determine whether % prolymphocytes was independently associated with survival was carried out in three stages, using Cox regression analysis. ≥10% prolymphocytes was an independent predictor of PFS in the first-stage model (n = 465), as were also randomization, gender, 11q deletion and TP53 deletion/mutation. In the second stage, ≥10% prolymphocytes was an independent predictor of OS when, in addition to the above first-stage variables, the model also included any one of the following: IGHV mutational status, B2M, CD38, ZAP70 or CLLU1 expression, NOTCH1 or SF3B1 mutations or telomere length. When all the significant variables from the second stage were included together in a final model (n = 121), % prolymphocytes was no longer an independent predictor of OS.

### Discussion

This is the first study to evaluate the clinical significance of increased circulating prolymphocytes in CLL within the context of a randomized chemotherapy trial and demonstrates that ≥10% prolymphocytes and an absolute prolymphocyte count of ≥15 × 10⁹/l are associated with a shorter PFS and OS in univariate analysis. Independent significance of ≥10% prolymphocytes is lost in multivariate analysis, although it retains significance for OS in models that include either IGHV mutational status or B2M, which we previously showed to be independent markers of outcome in the LRF CLL4 trial (Oscier et al, 2010). We also noted that patients with ≥10% prolymphocytes were significantly more likely to require second or third line treatments and to die from Richter transformation, although the latter observation requires confirmation in a larger study.

We were able to confirm in this large cohort of patients the previously documented associations between increased prolymphocytes, strong expression of Smlg and trisomy 12, and, for the first time, we document associations with male gender, elevated B2M, unmutated IGHV genes, high CD38 and CLLU1 expression, short telomere length, NOTCH1 mutations and absence of 13q deletion. Interestingly, and not expected, in a multivariate analysis only unmutated IGHV genes, high CD38 expression, NOTCH1 mutations and absence of 13q deletion, but not trisomy 12, were independently associated with ≥10% prolymphocytes. The association

### Table III. Variables associated with ≥10% prolymphocytes in multivariate analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio</th>
<th>Lower 95% CL</th>
<th>Upper 95% CL</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOTCH1 mutation</td>
<td>3.88</td>
<td>1.46</td>
<td>10.30</td>
<td>0.006</td>
</tr>
<tr>
<td>Absence of 13q deletion</td>
<td>4.41</td>
<td>1.82</td>
<td>10.69</td>
<td>0.001</td>
</tr>
<tr>
<td>Positive CD38 expression</td>
<td>6.48</td>
<td>1.44</td>
<td>29.25</td>
<td>0.02</td>
</tr>
<tr>
<td>Unmutated IGHV genes</td>
<td>5.02</td>
<td>1.39</td>
<td>18.17</td>
<td>0.01</td>
</tr>
</tbody>
</table>
with NOTCH1 mutations may become stronger once samples are screened for 3’ non-coding mutations (Puente et al., 2015).

Melo et al. (1986) raised the question as to ‘whether the phenomenon of prolymphocytoid transformation in CLL represents the release into the peripheral blood of cells in the mitotic phase of the cell cycle, or whether the prolymphocytes belong to a subclone with a growth advantage’. With regard to the first possibility, it has subsequently become clear that CLL cells recirculate between secondary lymphoid organs and peripheral blood and the latter contains subpopulations of resting or recently proliferating CLL cells which differ in their expression of surface receptors such as CD38, CD5 and CXCR4. (Calissano et al., 2011; Cuthill et al., 2015). Within secondary lymphoid organs, CLL cells divide within proliferation centres composed both of tumour cells and components of the tissue microenvironment, such as T cells, monocyte-derived nurse like cells and stromal cells, with which they interact. (Herishanu et al., 2011; ten Hacken & Burger, 2014). Tumour cells within proliferation centres contain medium-sized and large lymphoid cells comprising prolymphocytes and immunoblasts whose histological features mirror the morphology of circulating prolymphocytes and immunoblasts (Herreros et al., 2010). Immunohistochemical studies show that the large tumour cells within proliferation centres show increased expression of Ki67, CD20, CD23, CD38, IRF4, survivin (BIRC5), BCL2 and MYC compared to small lymphocytes outside proliferation centres, and upregulate NOTCH, CD40 and BAFF signalling pathways leading to NF-kB activation (Patten et al., 2008; Giné et al., 2010; Herreros et al., 2010; Gibson et al., 2015; Onaindia et al., 2015). Giné et al. (2010) studied tissue biopsies, mainly from lymph nodes, in 100 CLL patients of whom 73% had suspected Richter transformation. Twenty-eight per cent had expanded or confluent proliferation centres, which were associated with short survival. Ciccone et al. (2012) noted a similar association between confluent proliferation centres and short survival in a study of lymph node biopsies in 183 CLL patients. Neither of these studies reviewed lymphocyte morphology in the peripheral blood but, if the emergence of prolymphocytes into the blood represents the egress of cells from proliferation centres with a similar morphological appearance, then both a raised prolymphocyte count and an increase in a subpopulation with the immunophenotype of proliferating cells would be anticipated in these cases.

There are no data to confirm the second possibility that prolymphocytes represent a subclone. However, FISH analysis of proliferation centres and their surrounding areas of

Fig 2. Survival (A) Progression-free survival by <10% vs ≥10% prolymphocytes. (B) Progression-free survival by absolute prolymphocyte count. (C) Overall survival by <10% vs ≥10% prolymphocytes (D) Overall survival by absolute prolymphocyte count.
small lymphocytes showed a higher incidence of copy number abnormalities within the proliferation centres (Balogh et al., 2011). More recently, screening of concurrent blood and lymph node samples for genomic mutations and copy number chromosomal abnormalities identified cases with clonal driver mutations within lymph nodes that were only detected in the blood as small circulating subclones (Del Giudice et al., 2016). This supports the concept that mutations arise within a proliferative tissue compartment and raises the possibility that the circulating subclones may be enriched within cells with a 'proliferative' immunophenotype and/or those with prolymphocytic morphology.

The association between increased prolymphocytes and NOTCH1 mutations is a novel and unexpected finding. Circulating CLL cells with trisomy 12 have increased expression of the surface integrins CD11a, CD11b and CD18, which are down-regulated in the presence of NOTCH1 mutations (Riches et al., 2014). The rare cases of CLL with a MYC translocation (Huh et al., 2008; Put et al., 2012) have been shown to be associated with increased circulating prolymphocytes and although speculative, it is possible that these genomic abnormalities could facilitate the exit of prolymphocytes into the peripheral blood.

Regardless of the biology of prolymphocytes and the reasons for their appearance in the peripheral blood, our study confirms and extends earlier reports of their adverse prognostic significance. Although flow cytometric analysis has become an essential tool in the diagnostic evaluation of CLL and morphological expertise is less widespread, the distinction between cells with or without prominent nucleoli in a well-made blood film is relatively straightforward. Moreover, we noted that even ≥5% prolymphocytes were associated with a poorer outcome, and the higher cut-offs used in this study to define increased prolymphocytes clearly distinguish cases with typical morphology from those with CLL/PL. Depending on the clinical context, increased prolymphocytes at diagnosis or during the course of the disease may be a harbinger of progressive disease and may warrant further clinical and laboratory evaluation.

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**Author contributions**

DC was the principal investigator and takes primary responsibility for the paper; EM and DC evaluated lymphocyte morphology; RM performed flow cytometry, DO and JS undertook the core research on prognostic factors, ME performed the statistical analyses; DC, DO and ME wrote the paper.

**Conflict of interest**

The authors have no conflict of interests.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig S1.** Survival by % prolymphocytes (pl) within each treatment arm A: Progression-free survival B: Overall survival.

**Table S1.** Prolymphocyte data availability by patient/disease characteristics
References


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