RESEARCH LETTER

blood advances



TO THE EDITOR:

# Flow cytometry as a fast, cost-effective tool to assess *IGHV* mutational status in CLL

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With an incidence of 4.9 per 100 000 people per year in the United States, chronic lymphocytic leukemia (CLL) is one of the most common hematological diseases in Western countries.[1] Belonging to the group of indolent B-cell non-Hodgkin lymphomas, it typically affects older patients (median age at diagnosis 70 years) with a male predominance (sex ratio of 1.9:1) and displays a high clinical variability in its course.[1,2] Referring to the criteria proposed by the International Workshop on Chronic Lymphocytic Leukemia guidelines,[3] asymptomatic patients with early-stage disease (Binet A) should be monitored without therapy unless they have evidence of rapid disease progression. However, owing to the high variability of progression and numerous therapeutic advances, this classification has become insufficient to determine which patients will or will not benefit from an early treatment. Among the many prognostic factors identified,[4,5] the determination of immunoglobulin heavy chain (*IGHV*) mutational status is mandatory in progressive forms that will require treatment.[2] An unmutated status is associated with a more aggressive form of the disease with shorter survival,[6,7] but also with a poorer response to first-line chemo-immunotherapy FCR (rituximab combined with fludarabine and cyclophosphamide) because of a lack of sensitivity to fludarabine.[8,9] Knowing that the unmutated *IGHV*-status B clone originates from the proliferation of a naive pre–germ line B cell and that the mutated *IGHV*-status clone originates from the proliferation of a post–germ line memory B cell,[6,7] we hypothesized that a multiparametric flow cytometry (MFC) profile of the CLL clone would correlate with its mutational status. Physiologically, immunoglobulin D (IgD) is expressed by naive mature B cells and then disappears after lymph node passage due to class switching with an evolution into memory B cells.[10] Along with CD27, a marker of memory B cells,[11] we sought to distinguish 2 distinct B-cell profiles (naive IgD+/CD27− and memory IgD−/CD27+ B cells) and to correlate these data with the *IGHV* mutational status. Currently, the determination of this mutational status requires next-generation sequencing and is associated with a delay of several weeks for the results as well as a significant cost and requires dedicated bioinformatics tools.

Diagnostic CLL cells were obtained from cryopreserved peripheral blood. Each sample was washed twice in RPMI with 10% fetal bovine serum at 37°C and then stained for 30 minutes at room temperature with anti-CD5-FITC (clone REA782; Miltenyi Biotec), anti-IgD-PE (polyclonal; Agilent), anti-CD19-APC (clone REA675; Miltenyi Biotec), anti-CD27-AA750 (clone 1A4CD27; Iotest, Beckman Coulter), and anti-CD45-KO (clone J.33; Iotest, Beckman Coulter). Data acquisition was performed on a Navios flow cytometer and analyzed with Kaluza software (Beckman Coulter). The mutational status of rearranged *IGHV* genes was assessed as previously described.[12] This study was approved by the institutional review board of Amiens University Hospital and was conducted according to the Declaration of Helsinki.

The statistical review is detailed in the supplemental Data.[13]

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Data are available on request from the corresponding author, Thomas Boyer (boyer.thomas@chu-amiens.fr).

The full-text version of this article contains a data supplement.

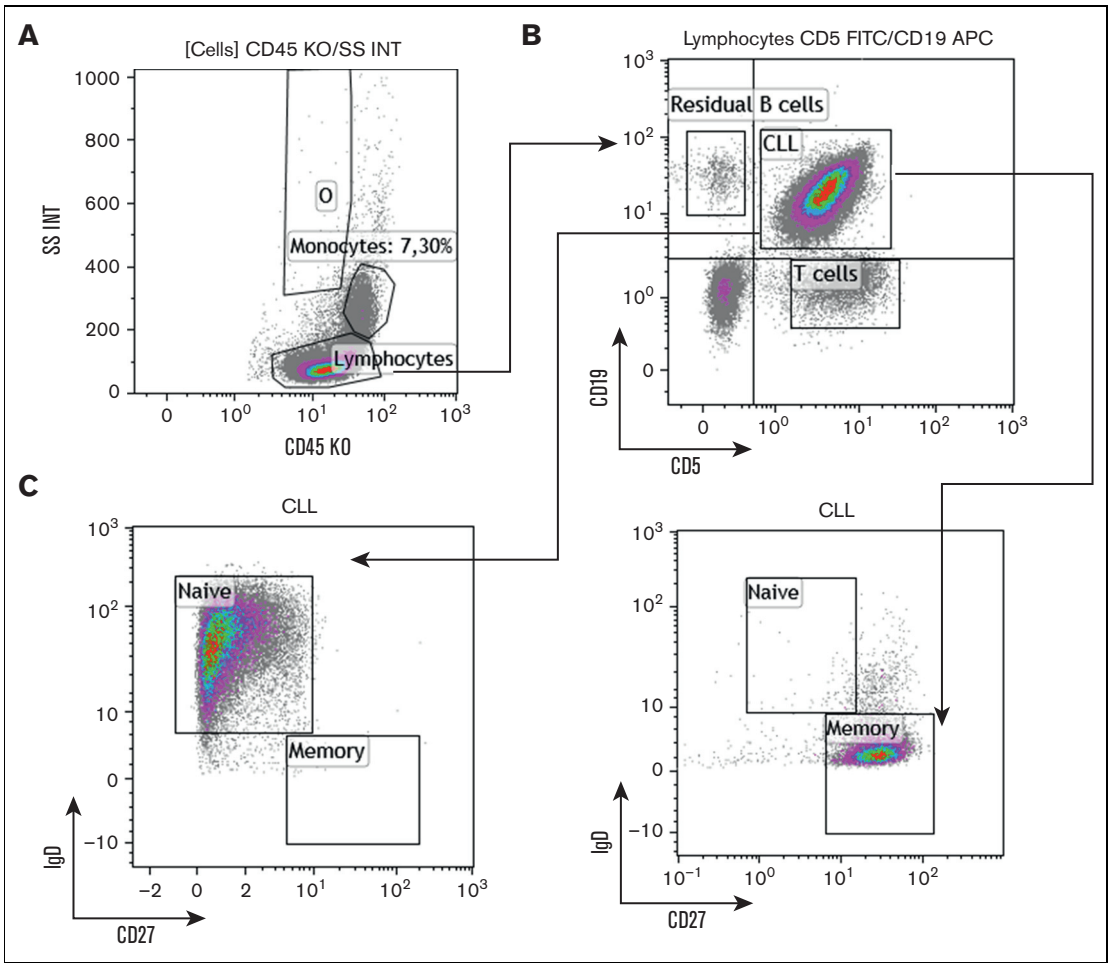


**Figure 1. Gating strategy and naive/memory B-cell profile in MFC.** (A) Lymphocytes were gated as CD45+/SS low cells. (B) Then, CLL cells were gated as CD19+/CD5+ lymphocytes. Normal residual B cells are CD19+/CD5− lymphocytes. (C) Naive (IgD+/CD27−) and memory (IgD−/CD27+) status of B-cell lymphocytes was finally determined.

A total of 98 patients were included in our study and were diagnosed with CLL between 2002 and 2021 at the University Hospital of Amiens. The median age at diagnosis was 65 years (range, 42-97). The sex ratio was 1.9:1; 82.7% (n = 81) of these patients were classified as Binet stage A, 11.2% (n = 11) stage B, and 6.1% (n = 6) stage C; and 52.6% (n = 52) of patients required therapy. Treatment included both chemotherapy, immunochemotherapy, anti-CD20 monoclonal antibody (such as rituximab or obinutuzumab) alone or in combination, and new biotherapies (ibrutinib, acalabrutinib, or venetoclax). During the follow-up, 31.6% of the patients received FCR and 21% bendamustine-rituximab. In addition, 22 of 57 (38.6%) treated patients relapsed on at least 1 first-line therapy. The *IGHV* mutational status was mutated/unmutated in 54.1% (n = 53) and 45.9% (n = 45) of the cases, respectively. The *TP53* abnormalities, either a del(17p) found on the karyotype and confirmed by fluorescent in situ hybridization and/or a *TP53* mutation, were present in 20.4% of the patients (n = 20).

Using MFC, we found a naive B-cell profile (IgD+/CD27−) in 43.9% (n = 43) of the cases and a memory B-cell profile (IgD−/CD27+) in the remaining 56.1% (n = 55). The gating strategy and representative plots of each profile are presented in Figure 1. For patients with a naive B profile, percentage of naive B clone ranged from 73.14% to 100% with a median of 96.28%. In addition, for patients with a memory B profile, percentage of clone B memory ranged from 51.92% to 100% with a median of 98.76%. Importantly, for all the naive profiles, IgD+/CD27 cells represented >70% of total cells. For the memory profile, in 50 of 55 patients, IgD−/CD27+ cells represented >80% of total cells. As reported in the literature, for the remaining 5 samples, IgD+/CD27+ cells were considered as memory cells.[11] The concordance between the determination of the *IGHV* mutational status by molecular biology and the MFC profile was 100% for the *IGHV* unmutated status/naive B-cell profile. Only 2 patients had a memory B-cell profile in MFC, whereas their *IGHV* mutational status was unmutated. When comparing between the 2 techniques, a sensitivity of 95.5% (95% confidence interval [95% CI], 85-99) and a specificity of 100% (95% CI, 93-100; $P < .001$) were obtained (Figure 2). For the purpose of internal validation, bootstrapped sensitivity and specificity for the *IGVH* unmutated status/naive B-cell profile were estimated 92.3% (95% CI, 83.7-98.1) and 95.7 (95% CI, 88.1-100), respectively.

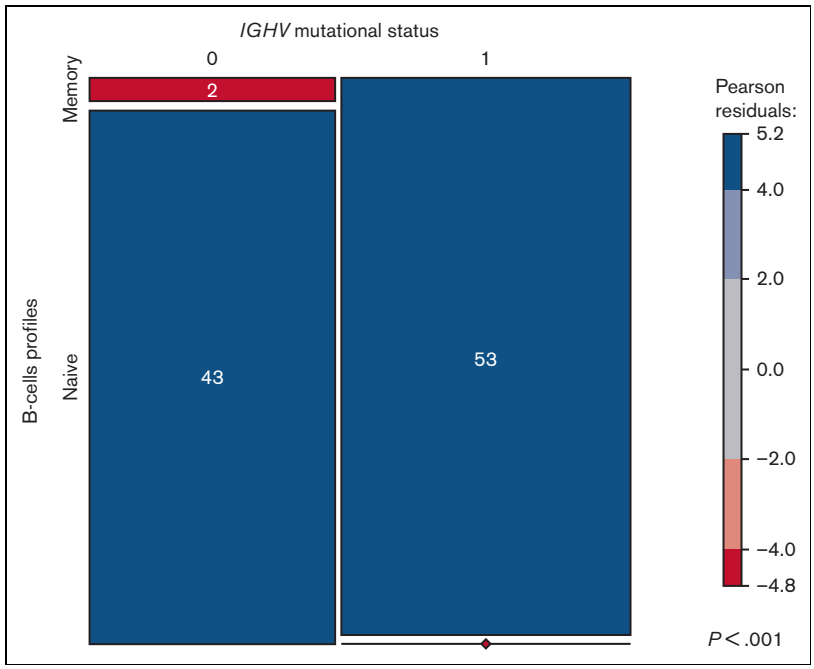


**Figure 2. Distribution of B-cell profiles according to *IGHV* mutational status.** Plot showing the distribution between naive B-cell or memory B-cell profiles according to *IGHV* mutational status (0 = unmutated; 1 = mutated).

In the external validation series, the crude sensitivity and specificity were estimated 92% (95% CI, 75-98) and 100% (95% CI, 86-100), respectively. The Bayesian posterior sensitivity was estimated 94% (95% CI, 88-98) and the posterior specificity 100% (95% CI, 100).

Here, we have demonstrated that the determination of naive B-cell/memory B-cell status by MFC on diagnostic samples was significantly correlated with the mutational status of the *IGHV* gene assessed by next-generation sequencing. The use of this new approach, with a reliable and robust tool, allows to orientate very quickly (in <24 hours) and in an inexpensive way, the therapeutic choice in patients with CLL. This determination, requiring only 2 additional antibodies (anti-IgD and anti-CD27), could easily be incorporated in the diagnostic panels of B-cell lymphomas along with the different markers allowing the calculation of the Matutes score. It is important to remember that the 98% cutoff is mathematical and that several clones, mutated and nonmutated, can coexist in the same patient. However, in our series, flow cytometry was not challenged by the coexistence of multiple clones, and double positivity (IgD+ CD27+) classified the lymphocytes as memory lymphocytes, as previously described.[11]

Nevertheless, MFC does not allow a study of the subclonal diversity of CLL (as well as the low-throughput Sanger sequencing technology). The next-generation sequencing methodology offers the possibility to detect the various subclones at diagnosis, and a recent study reported multiple unrelated clones in approximately 24% of patients with CLL, and this finding was associated with a worse prognosis.[14] We must acknowledge that this inexpensive, easily available method of assessment of mutational status provides no information on the heavy chain subset. However, given the marked efficacy of new targeted therapies in patients with unmutated CLL and in patients with *TP53* disruption, this information may be assessed for clinical routine only in remaining patients, that is, mutated CLL without *TP53* disruption. Furthermore, in the era of BTK inhibitors and BCL2-based therapeutic approaches, the prognostic importance of *IGHV* mutational status is to be put into perspective. Nonetheless, non-CT frontline treatment is not available everywhere, and this method may be very relevant in situations in which molecular biology cannot be performed (in lower-resource centers for instance).

Finally, medico-economic studies could complement these preliminary encouraging results.

**Contribution:** T.B., G.C., L.F., S.D., E.M., and V.H. performed immunophenotyping data acquisition; P.M., C.D., and J.-P.M. provided clinical and therapeutic data; and T.B., G.C., V.C., B.G., C.R., and L.G. wrote the manuscript, which was approved by all authors.

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