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Chronic Lymphocytic Leukemia

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CLL Biology and Prognosis

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Chronic lymphocytic leukemia (CLL) follows an extremely variable course with survival ranging from months to decades. Recently, there has been major progress in the identification of molecular and cellular markers that may predict the tendency for disease progression in CLL patients. In particular, the mutational profile of Ig genes and some cytogenetic abnormalities have been found to be important predictors of prognosis in CLL. However, this progress has raised new questions about the biology and prognosis of the disease, some of which are addressed here. Such questions include: 1) What is the role of the B-cell receptor (BCR) in CLL pathogenesis? 2) Is CLL one disease? 3) Is CLL an accumulative disease? 4) What is the normal counterpart of the CLL B lymphocyte? 5) Have the Rai and Binet staging systems become obsolete? 6) Which is the best surrogate for Ig mutational profiles?

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in Western countries and mainly affects elderly individuals. It follows an extremely variable course, with survival ranging from months to decades. Available treatments can often induce disease remission, though nearly all patients relapse, and there is consensus that CLL remains an incurable disease. The clinical diagnosis of CLL requires an absolute lymphocytosis with a lower threshold of greater than 5000 mature-appearing lymphocytes/μL. Nevertheless, it is common to find small percentages of larger or atypical cells, cleaved cells, or prolymphocytes. The routine availability of peripheral blood lymphocyte immunophenotyping has facilitated the diagnosis of CLL. Three main phenotypic features define B-CLL: the predominant population shares B-cell markers (CD19, CD2O, and CD23) with the CD5 antigen, in the absence of other pan-T-cell markers; the B cells are monoclonal with regard to expression of either κ or λ light chains and the B cells characteristically express surface immunoglobulin (slg), CD79b, CD20 and CD22 with low density. These characteristics are generally adequate for a precise diagnosis of CLL, and they also distinguish CLL from other disorders such as prolymphocytic leukemia, hairy-cell leukemia, mantle-cell lymphoma, and other lymphomas that can mimic CLL.[1]

CLL has previously been considered as a single entity with a variable clinical course. Recently, there has been considerable progress in the identification of molecular and cellular markers that may predict the tendency for disease progression in patients with CLL. Particularly, the mutational profile of Ig genes[2,3] and some cytogenetic abnormalities[4] have been demonstrated to display strong prognostic value. However, this progress has also introduced new questions in the fields of biology and prognosis. This update aims to discuss some selected unanswered questions in these different topics.

I. Unanswered Questions in CLL Biology

1) What is the role of the B-cell receptor (BCR) in CLL pathogenesis?

The BCR is a multimeric complex formed by the assembly of surface immunoglobulin (SIg) homodimer and the noncovalently bound heterodimer Igα/Igβ (CD79a/CD79b). Both these molecules play a key role in receptor expression and signal transduction through their immunoreceptor tyrosine-based activation motifs (ITAM), by linking the antigen binding Ig chains to intracellular tyrosine kinases of the Src-family. These events are transmitted through various signaling pathways into the cell nucleus to induce a cellular response.

Low expression of the BCR is the hallmark of the B-CLL lymphocyte.[5] It correlates with a reduced induction of

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protein tyrosine kinase activity that results in defective intracellular calcium mobilization and tyrosine phosphorylation, and leads to impaired responses of B-CLL cells when stimulated through the BCR pathway.[6,7]

The mechanisms accounting for poor expression of the BCR in CLL remain elusive. With the exception of one report describing a mutation in the Igβ molecule,[8] previous work has shown no genetic defects at the level of the BCR components.[9,10] In addition, there is normal transcription and intracellular synthesis of BCR components, which contrasts with their poor expression at the membrane level.[10,11]

Proteins comprising several subunits, such as the BCR, require folding and assembly. These processes take place in the endoplasmic reticulum (ER), where the proteins are modified (cleavage of signal peptide, N-glycosylation, formation of disulfide bonds) and folded before they pass into the Golgi apparatus. If folding and maturation are defective then the quality control system retains the defectively folded proteins and eliminates them. In the case of BCR, several chaperones, including calnexin, calreticulin, BiP and GRP94, have been shown to associate with the μ, CD79a and CD79b chains. Recent work from Vuillier et al demonstrated constant retention of the μ and CD79a chains in the ER of CLL B cells (**Figure 1a**; see Color Figures, page 550), leading to an impaired assembly of the BCR (**Figure 1b**; see Color Figures, page 550). This retention was frequently associated with defective glycosylation of both molecules, which paralleled the level of IgM expression on the surface of B cells (**Figure 1c**; see Color Figures, page 550). The impaired surface expression of IgM could not be accounted for by structural defects in BCR components and chaperone proteins.[5] However, the exact mechanism involved in defective assembly of the BCR is unclear. It also remains to be determined whether the CLL B lymphocyte is an anergic cell and whether BCR under-expression is related to the malignant transforming event. Evidence that the CLL cells may be anergic is suggested by the observation that similar underexpression of the BCR and defective signal transduction have been reported in the case of anergic murine B cells. This could be the consequence of BCR crosslinking by autoantigens, since the BCR of B-CLL can often be autoreactive.[7] The possibility also exists that BCR retention in the endoplasmic reticulum in CLL B cells could be accounted for by the blockade of its assembly through an interaction with an as yet uncharacterized intracellular protein, as reported for the K1 protein from human herpes virus 8 in a B-lymphoma cell line.[12] We believe that elucidation of the mechanisms implicated in BCR underexpression in B-CLL should be a key step in understanding disease pathogenesis.

The vast majority of B-CLL cells express CD5 and IgM/IgD and thus have a mantle zone-like phenotype of naive cells, which in normal conditions express unmutated Ig genes.[13] However, it has been shown that 50%-70% of CLL harbor somatic mutations of V_H genes[14] as if they had matured in a lymphoid follicle. Interestingly, the presence or absence of somatic mutations is associated with the use of particular V_H genes. Particular alleles of the V1-69[15] gene and the V4-39 gene display an unmutated profile.[16] A majority of members from the most prominent V_H3 family are expressed in a mutated form, whereas given the significant overexpression of V1-69 among V_H1 family members a majority of VH1 expressed genes display an unmutated profile (**Table 1**).[17] The fact that some genes like V_H1-69 and V_H3-07 recombine this V_H segment to particular JH segments and the restricted use of CDR3 sequences by CLLs expressing the V_H4-39 gene, suggest that the observed differences in BCR structure in B-CLL could result from selection by distinct antigenic epitopes.[16,18] It is presently unclear whether this putative antigen driven process occurs prior to leukemic transformation and/or that the precursors were transformed into leukemic cells at distinct maturational stages.

Recent studies analyzing signal competence revealed that unmutated CLLs (U CLLs) tended to express higher amounts of the BCR and respond better upon stimulation when compared to mutated CLLs (M CLLs). These results led to the proposal that unmutated CLLs keep their ability to respond upon BCR stimulation, whereas mutated ones resemble anergic B cells.[7] However, it remains to be determined whether anergy can induce the folding and assembly defects observed for CLL B cells.

Unexpectedly, two molecules with influence on the BCR tend to be more expressed among unmutated CLLs. The first one is the zeta-associated protein 70 (ZAP-70) a receptor associated protein tyrosine kinase, usually found in T and NK cells, but not in normal B cells. High levels of this protein are detected in the majority of unmutated CLLs.[19] Chen et al demonstrated that CLL B cells that express ZAP-70 are more likely to respond to IgM crosslinking with increased tyrosine phosphorylation and calcium flux than ZAP-70-negative CLL B cells.[20] This suggests that the expression of ZAP-70 in CLL allows more effective IgM signaling in CLL B cells, a feature that could contribute to the more aggressive course observed in these forms. The second molecule is the activation induced cytidine deaminase

Table 1. V_H expression according to Binet staging and mutational profile.

	V_H1	V_H2	V_H3	V_H4	V_H5	V_H6	TOTAL
Stage A							
Mutated	6	3	34	14	3	3	63
Unmutated	8	3	9	3	0	1	24
Total	14	6	43	17	3	4	87
Stages B + C							
Mutated	1	2	11	7	1	0	22
Unmutated	22	0	7	6	1	0	36
Total	23	2	18	13	2	0	58

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(AID), a B cell-restricted enzyme, required for somatic mutation and isotype switching. AID is upregulated, at least at the mRNA level, in unmutated CLL cells.[21,22] While there is evidence that AID expression could be confined to a small proportion of the clone,[23] it appears to be functional, since U-CLL cases can generate isotype-switched transcripts and protein and mutations in the pre-switch μ region.

2) Is CLL one disease or two diseases that look alike?

The mutational profile of Ig genes has been shown to be associated with disease prognosis.[2] These results suggest that CLL could correspond to two different diseases that share morphologic and phenotypic characteristics. In CLL with mutated Ig genes, the proliferating B cell may have transited through germinal centers, the physiologic site of hypermutation, whereas in CLL with unmutated Ig genes the malignant B cell may derive from a pre-germinal center naive B cell.

Supporting this view is the fact that the mutational profile of Ig genes is associated with specific genetic aberrations. Whereas 11q23 or 17p13 deletions are associated with poor outcome and with an unmutated V_H profile, 13q14 deletion or a normal karyotype are associated with a mutated profile. There is controversy as to whether trisomy 12 is associated to an unmutated status.[24,25] Although multiple instances of the disease in some families and the low incidence of the disease among individuals of Japanese origin, including those who migrated to Hawaii,[1] suggest that genetic influences are much stronger than environmental factors in the pathogenesis of the disease, the nature of this genetic predisposition remains unknown. None of the reported genetic aberrations is constant and many occur during evolution. In contrast with what is observed in other B cell malignancies, where chromosomal translocations associate oncogenes with Ig genes, in CLL the most frequent abnormalities are mutations, deletions or trisomies, whose biological significance remains unknown with the exception of those affecting the p53 suppressor gene.

Mutated and unmutated CLL patients clearly differ in terms of prognosis[2] and may also differ with respect to oncogenic mechanisms (11q deletions are almost always associated to an unmutated profile[4]). Despite these clinical and molecular differences, recent studies on gene expression profiling of B-CLL cells showed that CLL is characterized by a common gene expression signature that is independent of Ig mutational status and differs from other lymphoid cancers and normal lymphoid subpopulations, suggesting that CLL cells share a common mechanism of transformation and/or cell of origin.[19,26] These results are in agreement with the CLL monotonous phenotypic signature, i.e., BCR underexpression.

However, despite sharing a common signature CLLs expressing mutated and unmutated Ig genes differentially express more than 100 genes. Among these differentially expressed genes, overexpression of ZAP-70,[27] lipoprotein lipase (LPL), BCL-7a, dystrophin and gravin are observed in the aggressive unmutated cases,[19,26] while stable mutated cases overexpress Wnt3, CTLA-4, NRIP1 nuclear receptor gene, ADAM and the transcription factor TCF7.[19,26,28]

In addition, a non-supervised hierarchical clustering analysis is able to separate the stable mutated group from the aggressive unmutated one.[28] These results suggest that indolent mutated and aggressive unmutated CLLs constitute two variants of the same disease. The reason for the striking differences in clinical outcomes of these two variants remains unknown. Whether they correspond to differentiation stages at the moment of malignant transformation or whether unmutated forms of the disease are in a more activated form that favors the proliferative potential of the malignant clone still elude us. Better signaling transduction in the latter, when stimulated through the BCR pathway, and a positive role of ZAP-70 in this signalling could also play a role.[6,20]

3) Is CLL an accumulative disease?

The accumulation of mature B-cells that have escaped programmed cell death and have undergone cell cycle arrest in the G_0/G_1 phase is the hallmark of B-CLL. These cells have a low proliferative activity, and recent results support the hypothesis that an "in vivo" defective apoptosis accounts for accumulation of B cells in this disease. The importance of the anti-apoptosis bcl-2 oncogene in B-cell malignancies was established in studies of follicular lymphomas. These lymphomas carry the t(14;18), which places bcl-2 on chromosome 18 under the regulation of the Ig heavy chain enhancer on chromosome 14, leading to increased bcl-2 expression. In B-CLL cells, translocations of the bcl-2 gene are rare (less than 1% of cases) despite high levels of the bcl-2 protein. Its role in apoptosis inhibition is not clear since no correlation exists between "in vitro" apoptosis and the level of bcl-2 expression. Deregulation of cell cycle regulatory genes may also contribute to the accumulation of malignant cells in early phases (G_0/G_1) of the cell cycle. In B-CLL cells, elevated levels of the cyclin negative regulator $p27^{Kip1}$ protein are found in a majority of patients.[29] Given the key role of this protein in cell cycle progression, its overexpression in B-CLL cells could account for the accumulation of B cells in early phases of the cell cycle. Taken together, these data suggest that CLL is a disease resulting from accumulation rather than from proliferation. However, in contrast with in vivo results, apoptosis occurs after in vitro culture, suggesting a role of the microenvironment in B-CLL cell survival.[30] Reports indicating that apoptosis in vitro is prevented by exposure to interleukin (IL)-4 as well as by stimulation via surface CD40, also favor this view. In vivo, such inhibition may occur in pseudo-follicles observed in the lymph nodes and in the cell clusters described in the bone marrow.[30] These pseudo-follicles are not simply a collection of monoclonal B cells but include increased numbers of CD4 T cells in close contact with proliferating B cells and expressing CD40

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ligand. These activated CD4 T cells could be recruited by tumor B cells since they constitutively express the T cell-attracting chemokines CCL17 and 22.[7] This could be in agreement with a model of selective survival of certain clonal sub-members, which would receive survival signals in these particular sites. By using a non-radioactive, stable isotopic labelling method to measure CLL kinetics, Messmer et al demonstrated that B-CLL is not a static disease that results simply from accumulation of long-lived lymphocytes, but a disease where a dynamic process in which cells proliferate and die, often at appreciable levels.[31] This finding is in conflict with the dogma that B-CLL is a disease characterized almost exclusively by cell accumulation due to a defect in apoptosis. This mechanism may compensate for the clonal decrease that could occur in the periphery by apoptosis and depending on its importance could play a major role in the regulation of the tumor burden.

4) What is the normal counterpart of the CLL B lymphocyte?

It has been proposed that the normal counterpart of leukemic cells may be a follicular mantle-zone B1 cell, which normally expresses CD5, CD23, is negative for CD38, co-expresses membrane IgM and IgD and utilizes unmutated Ig V region genes. In contrast, germinal center B cells express CD38, IgD, frequently exhibit isotype switching and display somatic mutations in Ig genes.[13]

However, CLL B cells, whether displaying mutated or unmutated Ig V genes, almost always express IgM and IgD, CD5 and CD23. In addition, CD38 expression paradoxically predominates among unmutated cases,[2] and active class switch recombination (CSR) at the RNA and protein level is often observed among CLL B cells.[21,22] Constitutive expression of activation-induced cytidine deaminase (AID) predominates among CLL B cells displaying active CSR and unmutated V genes. This enzyme is known to play a key role in both CSR and somatic hypermutation, and is not usually expressed in normal circulating B cells, but is constitutively expressed in germinal center B cells. Since expression of AID is induced upon CD40 ligand stimulation,[21] its frequent expression by CLL B cells displaying unmutated V genes challenges the notion that these correspond to naive B cells.

As proposed by Chiorazzi et al,[16,32] it is also possible that the normal counterpart of B-CLL cells could be marginal zone B cells, which have been demonstrated to express both mutated and unmutated V genes. Although marginal zone B cells do not express CD5, this expression could be the consequence of the activation profile of CLL B cells. However, this provocative hypothesis still fails to explain the common gene profiling signature in B-CLL.

II. Unanswered Questions in CLL Prognosis

1) Is it time to move away from the Rai and Binet staging systems?

In CLL, one-third of patients never require treatment and have a long survival; in another third, an initial indolent phase is followed by disease progression; the remaining third patients exhibit an aggressive disease at the onset and need immediate treatment.[1] The development of the Rai and Binet staging systems has allowed the division of patients with CLL into three prognostic groups: good, intermediate and poor prognosis. Binet's good prognosis group (stage A, 63% of CLL patients with a 10-year survival of 51%) includes twice as many patients as Rai's Stage 0, since it includes all Rai's 0, 2/3 of Rai's I and 1/3 of Rai's II. Rai's stage 0, which includes 31% of CLL patients, display a 10-year survival of 59%. Rai's intermediate prognosis group includes 59% of CLL patients compared to 30% in the Binet's intermediate group.[33] These staging systems provided a foundation that allowed clinicians to design therapeutic strategies for the disease.

The low-risk group (Stage A from Binet or Stage 0 from Rai), has a median age at diagnosis of 64 years and an expected survival of >10 years, which is close to the life expectancy of a normal population matched for sex and age. However, over 25% of these indolent cases die of causes related to CLL, 40% progress to advanced stages and 50% ultimately require treatment (**Table 2**).[33] These results demonstrate that neither the Rai nor the Binet staging system are able to accurately predict which patients among the good prognosis group will develop progressive disease. To develop risk-adapted therapeutic strategies, better information that allows prediction of the clinical course of disease at initial diagnosis is needed. Lymphocyte doubling time, serum levels of β2-microglobulin, thymidine kinase and soluble CD23, as well as CD38 expression on malignant cells can help predict disease activity (reviewed in [34]).

Since CLL B cells display a phenotype similar to naive B cells from the mantle zone, it is logical to assume that they should express unmutated Ig V genes. Following an initial description indicating that at least half of CLLs were expressing V genes containing numerous somatic mutations,[14] Hamblin et al and Damle et al made a seminal observation indicating that the clinical behavior of CLL is related to the mutational status of immunoglobulin (Ig) genes.[2,3] CLL with mutated Ig genes displays a good prog-

Table 2. Long-term results of Binet A and Rai 0 patients from the abstention arm of the CLL-80 trial.[33]

	% of Patients	10-Year Survival (%)	% of Patients Without Evolution	% of CLL-Related Deaths	% of Patients Evolving to B or C	% of Patients Requiring Treatment
Stage 0	31	59	57	27	32	43
Stage A	65	51	47	31	41	53

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nosis, whereas those with unmutated Ig genes have a poor prognosis. Since then this observation has been extensively confirmed[24,25,35] and there is general agreement that the mutational status of Ig genes constitutes a very strong prognostic indicator in CLL (**Table 3**). We recently confirmed the strong prognostic value of V_H mutational profile in a retrospective series of 146 patients with a long follow-up.[2,35] Our results also showed that the mutational profile of Ig genes is able to delineate prognostic groups within all Binet's stages. Since mutated and unmutated groups display different prognoses when comparing A with B/C cases, both Binet's staging and V_H genes retain their independent prognostic significance in CLL (**Table 3**). Indeed, they should be most likely complementary.[17]

Interestingly, the rearrangement of a specific VH gene, the V3-21 has been associated with poor prognosis whether mutated or not and to the expression of highly restricted VL usage and homologous CDR3.[36] Curiously, the incidence of the V3-21 gene in CLL appears higher in certain geographical regions,[37] and this could reflect either a different pathogenesis or variable bias in the aging repertoire.

The presence in the leukemic B cells of cytogenetic abnormalities like 11q or 17p deletions[4,24,25] also constitute a strong prognostic indicator in CLL (**Table 4**). Döhner et al demonstrated in a extensive series of 325 CLL patients that chromosomal aberrations can be detected by fluorescence in situ hybridization (FISH) in 82% of cases.[4] The most frequent alteration is the 13q deletions that are observed in 55% of patients, followed by the 11q deletion and trisomy 12, whereas 17p deletion involving the p53 protein is observed less frequently. Interestingly, the presence of a 17p or 11q deletion is associated with poor prognosis and predominates among advanced stages of the disease and among patients displaying unmutated V genes, whereas the 13q is associated with good prognosis, initial stages of the disease and with a mutated profile of Ig V genes (**Table 4**).

The V_H mutational profile has the advantage that it remains constant during clonal evolution, which contrasts with genomic aberrations and serum markers. In addition, since 11q23 or 17p13 deletions are associated with poor outcome and an UM V_H profile in about one-third of unmutated CLL, they could be considered as a subgroup of the unmutated group. Although CD38 expression is associated with poor prognosis, its relationship to Ig mutational status remains controversial.[2,24,25,38] Besides the fact that its expression has been demonstrated to change during disease evolution, there are important concerns related to inter-laboratory variations, the definition of the best cut-off value, and assessment of its intensity of expression.[34,38]

Table 3. Relationship between Binet staging, mutational status of Ig genes and expression of ZAP-70, lipoprotein lipase (LPL) and ADAM-29 genes.

	V_H MUT	V_H UNM	ZAP-70⁻	ZAP-70⁺	LPL⁺	ADAM 29⁺
Stage A						
%	69	31	65	35	31	69
OS, mo.*	NA	87				
PFS, mo.	156	42	NA	29	87	NA
Stages B+C						
%	35	65	31	69	61	39
OS, mo.	120	78	100	80	65	100

* Due to the relatively short follow-up in these series and the small number of events overall survival could not be performed for ZAP-70, LPL and ADAM-29 expression among stage A patients.
Abbreviations: OS, overall survival; PFS, progression-free survival

Table 4. Genetic aberrations in chronic lymphocytic leukemia (CLL), according to Döhner et al.[4]

CLL Patients	Normal	13q Alone	12q Trisomy	11q Deletion	17p Deletion
%*	18	55	16	18	7
Overall Survival, mos.	120	132	120	84	30
% Stage A	53	72	51	25	23
% Stage B	30	20	34	50	41
% Stage C	17	8	15	25	36

* Percentages within Binet stages refer to the frequency with which each cytogenetic profile is observed in the different stages.

2) Which is the best surrogate for Ig mutational profiles?

Since sequencing Ig V genes is costly, time consuming and inaccessible for most medical facilities, the detection of appropriate, reliable surrogate markers for *IgVH* mutational status has attracted worldwide attention. Damle et al suggested that CD38 determination might be a useful alternative.[2] However, correlation of Ig mutational status with CD38 expression remains controversial, though its independent prognostic value is demonstrated.[24,25] In addition, a clear correlation between mutational profiles and serum levels of thymidine kinase has also been reported.[39] Following the initial description that ZAP-70 is overexpressed among CLLs expressing unmutated V genes,[19] Crespo et al[40] proposed that detection of ZAP-70 by a simple and convenient multiparameter flow-cytometric test, is highly correlated with the presence of an unmutated profile of Ig genes (91% sensitivity). This has been presently confirmed in other studies.[27,41] In a study of gene expression profiling performed on 18

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CLL cases, we identified the lipoprotein lipase (LPL) and ZAP-70 genes as being consistently overexpressed among unmutated CLL and the metalloproteinase ADAM 29 among mutated CLLs (**Table 3**).[28] We quantified expression of *LPL* and *ADAM29* genes by PCR, and ZAP-70 protein by flow-cytometry in a cohort of 127 CLL patients, and evaluated the correlation between *IgVH* mutational status and clinical outcome. Combining *LPL* and *ADAM29* mRNA quantifications by a simple 1 to 1 ratio (L/A ratio) provided a 90% concordance rate with the *IgVH* mutational status. Simultaneous usage of the L/A ratio and ZAP-70 expression allowed an almost perfect (99%) assessment of the *IgVH* status in the 80% of patients with concordant results (L/A^+, $ZAP\text{-}70^+$ or L/A^-, $ZAP\text{-}70^-$). *IgVH* mutational status, ZAP-70 and the L/A ratio were predictive of event-free survival for the whole cohort and for stage A patients. In contrast to ZAP-70, which failed to segregate advanced forms of the disease, the L/A ratio was an independent prognostic factor for stage B and C patients (**Table 3**).

The association of Binet's stage A with a mutated V_H profile (**Table 3**) isolates a group with very good prognosis, accounting for more than 40% of CLL patients (12-year survival of 75%, median progression-free survival of 13 years and <10% CLL related deaths). The survival of this group of patients should be very close to that of a sex- and age-matched normal population. However, even in this very good prognosis group, progression is observed for one-third of these patients and CLL-related death for 10% of them. Since it is possible that such progression depends on a second oncogenic event, it is unclear whether it is possible to identify the small group of stage A mutated patients who will display disease progression.[1,33] This group mostly includes the CLL patients who will not progress and will die of causes unrelated to CLL (about 1/3 of CLL cases). The addition of biological prognostic indicators such as the mutational profile of Ig genes and cytogenetic abnormalities allows better identification of initial forms of the disease (stages A and O) that will progress during evolution (about 1/3 of CLLs). Cells from such stage A patients usually display unmutated Ig genes and/or cytogenetic abnormalities such as 11q or 17p deletion.

In conclusion, the recognition of novel biological variables has had a major impact on our understanding of CLL. Some of them appear to be of considerable prognostic importance but as yet there is no available evidence to suggest that changing therapeutic approaches on the basis of these results will lead to an improvement in outcome. There is a pressing need for prospective clinical trials to address the stratification of patients according to these factors.

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