Chronic lymphocytic leukaemia

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Chronic lymphocytic leukaemia is the commonest form of leukaemia in Europe and North America, and mainly, though not exclusively, affects older individuals. It has a very variable course, with survival ranging from months to decades. Major progress has been made in identification of molecular and cellular markers that could predict disease progression in patients with chronic lymphocytic leukaemia. In particular, the mutational profile of immunoglobulin genes and some cytogenetic abnormalities are important predictors of prognosis. However, these advances have raised new questions about the biology, prognosis, and management of chronic lymphocytic leukaemia, some of which are addressed here. In particular, we discuss how better understanding of the function of the B-cell receptor, the nature of genetic lesions, and the balance between proliferation and apoptosis have affected our ability to assess prognosis and to manage chronic lymphocytic leukaemia. Available treatments generally induce remission, although nearly all patients relapse, and chronic lymphocytic leukaemia remains an incurable disease. Advances in molecular biology have enhanced our understanding of the pathophysiology of the disease and, together with development of new therapeutic agents, have made management of chronic lymphocytic leukaemia more rational and more effective than previously. Unfortunately, we know of no way that chronic lymphocytic leukaemia can be prevented. Early detection is practised widely, but seemingly makes no difference to the patient’s eventual outcome.

Epidemiology

The incidence of chronic lymphocytic leukaemia varies with the age and sex structure of the population. Analysis of the Surveillance, Epidemiology, and End Results (SEER) database notes the US incidence as being 3·5 per 100 000 per year (men 5·0, women 2·5).1 In the Leukaemia Research Fund data collection study, researchers gathered data from individual haematologists responsible for laboratories covering about a third of the population of England and Wales and reported an incidence of chronic lymphocytic leukaemia in the UK of 6·15 per 100 000 per year, although this value concealed a variation between 1·3 and 13·7 per 100 000 per year in different health districts, dependent largely on how assiduous is the case finding. According to SEER data, the median age for diagnosis of the disease is 70 years for men and 74 years for women, and median age at death is 76 years and 81 years, respectively. White American individuals have a slightly higher incidence than those of African-American origin (3·9 vs 2·8 per 100 000 per year), but in American people of Chinese, Japanese, and Filipino extraction, incidence is about five times lower, even in those who have adopted a fully American lifestyle.2 Early data put the incidence of chronic lymphocytic leukaemia in Jewish people at twice that of non-Jewish North American individuals.3

Chronic lymphocytic leukaemia can arise in families.4–6 First-degree relatives of patients with the disease are three times more likely to have chronic lymphocytic leukaemia or another lymphoid neoplasm than the general population.7 With a four-colour flow-cytometric assay, Rawstron and colleagues8 noted that 3·5% of healthy individuals older than 40 years had a population of monoclonal lymphocytes in their blood, with the immunophenotypic characteristics of chronic lymphocytic leukaemia cells, at concentrations lower than 3·5×10⁹/L; in first-degree relatives of patients with familial chronic lymphocytic leukaemia, the prevalence of such cells is between 13·5% and 18%.9,10 The relation between subclinical chronic lymphocytic leukaemia and full-blown disease is a matter of intense investigation in several laboratories. No consistent evidence is available to link chronic lymphocytic leukaemia with environmental exposure to either radiation or chemicals, except in the case of agricultural workers and herbicides. On Jan 23, 2003, the US National Academy of Sciences’ Institute of Medicine published a report concluding that there is “sufficient evidence of an association between exposure to Agent Orange, a herbicide used in Viet Nam, and the development of chronic lymphocytic leukaemia”.11 Although ionising radiation has traditionally been absolved from causing chronic lymphocytic leukaemia, recent studies have suggested that this may be unwarranted.12

Diagnosis

Clinical diagnosis of chronic lymphocytic leukaemia is defined by absolute lymphocytosis of at least 5×10⁹/L.
mature-appearing lymphocytes and an appropriate immunophenotype (figure).13 These characteristics distinguish chronic lymphocytic leukaemia from mantle-cell lymphoma and splenic marginal-zone lymphoma, the diseases that most frequently mimic chronic lymphocytic leukaemia.14 In a few individuals, tumour is confined to lymph nodes or other tissues without blood or bone marrow involvement. In these people, the disorder is known as small lymphocytic lymphoma: histological findings and immunophenotype are identical to chronic lymphocytic leukaemia and management should be the same.15 Individuals without involvement of lymph nodes or other tissues, who have a population of small lymphocytes immunophenotypically similar to chronic lymphocytic leukaemia cells in blood or bone marrow below the threshold necessary for diagnosis of chronic lymphocytic leukaemia, are designated as having monoclonal B-cell lymphocytosis.16

Molecular and cellular markers have been identified that could predict disease progression. In particular, the mutational profile of immunoglobulin genes7,28 and some cytogenetic abnormalities29 show strong prognostic value. However, these biological differences do not separate chronic lymphocytic leukaemia into two different disorders; it remains one disease with heterogeneous features.30

**Pathophysiology**

Despite the ready availability of tumour cells in chronic lymphocytic leukaemia, up to now, very little has been known about the pathophysiology of the disease. The cells themselves are remarkably inert in vitro. Most, if not all, cell lines attributed to chronic lymphocytic leukaemia are either from patients with mantle-cell lymphoma masquerading as chronic lymphocytic leukaemia or B-cell lymphoblastoid lines from contaminating normal lymphocytes.21 Until the past few years, no animal model had existed for chronic lymphocytic leukaemia. The TCL1 transgenic mouse develops a CD5+ B-cell lymphoproliferative disease that serves as a model for aggressive forms of chronic lymphocytic leukaemia but not for the frequent indolent form.24

The discovery that the mutational status of \textit{IGHV} genes affects profoundly the prognosis of chronic lymphocytic leukaemia has acted as such a spur to our understanding of the disease’s pathology, but full review of this topic is not possible within the space confines of this Seminar. Instead, we will concentrate on three topics: the B-cell receptor; genetic abnormalities revealed by interphase cytogenetics; and the balance between proliferation and apoptosis.

**The B-cell receptor**

The B-cell receptor is a multimeric complex formed by the assembly of a surface immunoglobulin homodimer and a non-covalently-bound heterodimer Iga/Igb (CD79A/CD79B). Low expression of the B-cell receptor is the hallmark of lymphocytes in chronic lymphocytic leukaemia.21

The mechanisms accounting for poor expression of the B-cell receptor in chronic lymphocytic leukaemia remain elusive. With the exception of one report of mutation in CD79B,25 no genetic defects in B-cell-receptor components have been recorded.21,26 By contrast with their poor expression at the membrane level, transcription and intracellular synthesis of components of the B-cell receptor are normal,24,25 but they cannot be assembled and transported from the endoplasmic reticulum to the cell surface because of a folding and glycosylation defect of the \( \mu \) and CD79A chains, although not of the CD79B chain. Poor expression of the CD22 molecule in B-cell chronic lymphocytic leukaemia cells was also shown to result from a folding defect arising in CD79A.25

Most B-cell chronic lymphocytic leukaemia cells express CD5 and IgM/IgD and, thus, have a mantle zone-like phenotype of naïve cells that, in normal conditions, express unmutated immunoglobulin genes.24 However, 50–70% of cases of chronic lymphocytic leukaemia have somatic mutations of \textit{IGHV} genes,3 as if they had matured in a lymphoid follicle. Presence or absence of somatic mutations is associated with particular \textit{IGHV} genes. Specific alleles of the \textit{IGHV1-69} gene30 and the \textit{IGHV4-39} gene have an unmutated profile.31 Subsequently, workers have reported32 that more than 20% of patients with chronic lymphocytic leukaemia carry stereotypic B-cell receptors.32–34 Of note, the \textit{IGHV3-21} gene shows strikingly homologous \textit{IGHV} and \textit{IGLV} gene rearrangements and is associated with poor prognosis, whether expressed in a mutated or unmutated form.35,36 These results strongly suggest that a common antigen epitope is recognised by these highly homologous molecules. With respect to the epitope recognised, research has shown that unmutated chronic lymphocytic leukaemia cells express highly polyreactive antibodies, whereas most mutated ones do not.37,38 Infections with encapsulated organisms might be a trigger for development of chronic lymphocytic leukaemia, and work has shown that individuals with a history of pneumonia are significantly more likely to develop chronic lymphocytic leukaemia than are people without this history, and that the risk increases with number of attacks.39,40
When stimulated through the B-cell receptor pathway, the response of chronic lymphocytic leukaemia cells is impaired. Low expression of the B-cell receptor correlates with reduced induction of protein tyrosine kinase activity and defective intracellular calcium mobilisation and tyrosine phosphorylation. Individuals with differing responses to IgM ligation, related to *IGHV* gene status. Findings of one study showed that chronic lymphocytic leukaemia cells expressing unmutated *IGHV* genes had a better response in most cases to stimulation via the B-cell receptor than did cells expressing mutated *IGHV* genes. Unexpectedly, high amounts of ZAP70—a receptor-associated protein tyrosine kinase usually found in T cells and natural killer cells but not in normal circulating B cells—are detected in most patients with unmutated chronic lymphocytic leukaemia. Chronic lymphocytic leukaemia B cells that express ZAP70 are more likely to respond to IgM crosslinking with increased tyrosine phosphorylation and calcium flux than are those that do not express ZAP70. This effect might happen for any or all of the following reasons. First, after B-cell receptor ligation, ZAP70 undergoes tyrosine phosphorylation and becomes associated with surface immunoglobulin and CD79B. Second, ZAP70 mediates inhibition events that terminate the signalling response. Finally, ZAP70 expression is associated with advantageous survival responses because of enhanced access to proliferation centres. Altogether, expression of ZAP70 in chronic lymphocytic leukaemia allows effective IgM signalling in B cells, which might lead to an aggressive disease course.

The apparently anomalous expression of ZAP70 in chronic lymphocytic leukaemia cells is not accounted for completely. Heat-shock protein 90 (HSP90) is a molecular chaperone that catalyses the conformational maturation of many signalling proteins in cancer, known collectively as clients. With inhibitors of HSP90, Castro and colleagues detected that ZAP70 is a client protein in tumour cells, but not in T cells, from patients with ZAP70-positive chronic lymphocytic leukaemia, suggesting that the presence of ZAP70 is an oncogenic event. On the other hand, ZAP70 is expressed at various stages of B-cell maturation and in other B-cell malignant diseases. It is present in normal pre-B cells and pro-B cells and in acute leukaemias derived from them. By studying normal tonsillar cells, Nolz and coworkers and Cutrona and colleagues detected ZAP70-positive B cells, concentrated particularly in germinal centres. ZAP70 seems to be coexpressed with other activation markers. In chronic lymphocytic leukaemia, higher amounts of ZAP70 are expressed by lymph-node cells than by circulating cells. In turn, high levels of ZAP70 expression lead to increased sensitivity to chemokine migratory signals. Whether expression of ZAP70 in chronic lymphocytic leukaemia cells is a result of frequent visits to proliferation centres or is the cause of enhanced access to them is still not clear.

Another unexpected molecule expressed by a subset of chronic lymphocytic leukaemia B cells is CD38. In the B-cell compartment, CD38 is not a lineage marker, but this molecule is expressed at times during B-cell development when cell-to-cell interactions are crucial. Examples include an early bone-marrow precursor cell, cells in the germinal centre, and plasma cells. In chronic lymphocytic leukaemia, expression of CD38 predominates in patients with unmutated *IGHV* genes and is associated with poor prognosis. Expression of CD38 in chronic lymphocytic leukaemia B cells favours B-cell growth and survival through sequential interactions between CD38 and CD31 and between CD100 and plexin B1 (PLXNB1).

The activation-induced cytidine deaminase (AICDA), a B-cell-restricted enzyme needed for somatic mutation and isotype switching, is upregulated in unmutated chronic lymphocytic leukaemia cells. Although evidence exists that AICDA expression could be confined to a small proportion of the clone, AICDA seems to be functional, since unmutated cases of chronic lymphocytic leukaemia can generate isotype-switched transcripts and proteins and mutations in the pre-switch μ region. Upregulation of AICDA could be associated with loss of target specificity, resulting in mutations in non-immunoglobulin genes such as *BCL6*, *MYC*, *PAX5*, and *RHOH*, which are linked to aggressive disease.

**Genetic abnormalities**

Although multiple instances of chronic lymphocytic leukaemia in some families, and low frequency of the disease in individuals of Japanese origin, suggest that genetic effects might be stronger than environmental factors in the pathogenesis of chronic lymphocytic leukaemia, the nature of this genetic predisposition remains uncertain and different genes may be involved in different families. A recent paper has identified a family in which a single nucleotide polymorphism down-regulates the expression of *DAPKI* transcription. The same polymorphism has been identified in one sporadic case of chronic lymphocytic leukaemia, but not in other familial cases. DAPKI is a pro-apoptotic protein whose expression is also suppressed in sporadic cases of chronic lymphocytic leukaemia by an epigenetic mechanism. The many unsuccessful attempts to establish genetic linkages have been reviewed by Goldin and Slager. None of the reported genetic aberrations is constant, and whether they constitute initial events or arise during evolution is presently unclear. By contrast with observations in other B-cell malignant diseases, which typically exhibit balanced chromosomal translocations, in chronic lymphocytic leukaemia the most frequent abnormalities are mutations, deletions, or trisomies. Translocations do arise but are usually unbalanced, resulting in loss of genetic material.
the past few years have cytogenetic techniques been developed that make this proposition feasible. Döhner and colleagues showed in a series of 325 patients with chronic lymphocytic leukaemia that chromosomal aberrations can be detected in interphase cells by fluorescence in-situ hybridisation (FISH) in 82% of cases. The most frequent alterations are a deletion on chromosome 13q (55%), trisomy 12 (18%), and a deletion on chromosome 11q (16%). A deletion on chromosome 17p, affecting the TP53 protein, is seen less frequently (7%). The presence of a 17p or 11q deletion is associated with poor prognosis and predominates in advanced stages of chronic lymphocytic leukaemia and in patients with unmutated IGHV genes, whereas the 13q deletion or a normal karyotype are associated with good prognosis, initial stages of the disease, and mutated IGHV genes (table 1). Controversy exists about whether trisomy 12 is associated with an unmutated status and poor prognosis.

Patients with mutated and unmutated chronic lymphocytic leukaemia differ clearly in terms of prognosis (see also section on Assessment of prognosis).

Genetic lesions associated with deletions of the short arm of chromosome 17 (del17p13), which encodes the TP53 tumour-suppressor gene, and the long arm of chromosome 11 (del11q23), which encodes the ataxia telangiectasia mutated (ATM) gene, result in a loss of function of TP53. TP53 is a transcription factor activated by strand breaks in DNA. It can trigger apoptosis or cell-cycle arrest. Thus, by controlling repair or elimination of cells with damaged DNA, TP53 maintains the integrity of the genome and prevents clonal progression. ATM is a kinase that regulates TP53. Many cytotoxic drugs require the ATM/TP53 pathway to be intact for them to be effective. A simple screening test that assesses how intact this pathway is has been described. Defects in the ATM/TP53 pathway constitute the strongest independent predictors for disease that is resistant to standard treatment.

Deletions of the ATM gene do not produce such a severe syndrome as do deletions of TP53, with some patients having a fairly benign disease course. Possibly, for ATM function to be impaired, mutations are necessary on the other ATM allele. Conversely, Kalla and co-workers have identified other genes affecting regulation of the cell cycle and apoptosis—namely NPAT, CUL5, and PPP2R1B—in the commonly deleted 11q22-q23 segment, which might underlie the severity of the chronic lymphocytic leukaemia.

The pathogenic role of trisomy 12 in chronic lymphocytic leukaemia remains unresolved. CLLU1—the first disease-specific gene identified in people with chronic lymphocytic leukaemia—has been located to 12q22, but there seems to be no difference in protein expression in patients with or without trisomy 12.

MicroRNA molecules (miRNAs) have an important role in regulation of gene expression during human development. Using a microarray containing hundreds of human precursors and mature miRNA oligonucleotide probes, Calin and colleagues recorded significant differences in miRNA expression between chronic lymphocytic leukaemia B cells and normal CD5+ cells. They showed the absence of two miRNAs (miR15 and miR16) associated with mutations in expressed IGHV genes and with deletions in the 13q14 region. As part of normal control of gene expression, miR15 and miR16 seem to target BCL2, and their absence in chronic lymphocytic leukaemia could be a major factor in prevention of apoptosis. However, Fulci and coworkers have been unable to confirm these findings. They reported low expression of these miRNAs in only 12% of patients, despite 58% having del13q14. All individuals with a deletion of both 13q14 allels showed striking miR15a downregulation, but such pronounced downregulation of both miRNAs was not paralleled by any significant increase in BCL2 expression levels. Fulci’s team also noted overexpression of miR150, miR223, and miR29c in IGHV-mutated chronic lymphocytic leukaemia compared with unmutated cases.

Despite clinical and molecular differences, chronic lymphocytic leukaemia is characterised by a common gene-expression signature that differs from other lymphoid cancers and normal lymphoid subpopulations, suggesting that patients with the disease—in agreement with the monotonous phenotypic signature—share a common mechanism of transformation, cell of origin, or both. However, despite sharing a common signature, chronic lymphocytic leukaemias expressing mutated and unmutated IGHV genes differentially express more than 100 genes. Of these, overexpression of genes encoding ZAP70, lipoprotein lipase (LPL), BCL7A, dystrophin (DMD), and gravin (AKAP12) are noted in individuals with aggressive unmutated disease, whereas people with stable mutated chronic lymphocytic leukaemia overexpress WNT7, CTLA4, the gene encoding nuclear receptor interacting protein 1 (NRIP1), ADAM29, and TCF7. Furthermore, evidence suggests that for particular IGHV genes, such as IGHV1-69 and IGHV3-21, different genomic aberrations might be associated with differential gene expression. These results indicate that indolent mutated and aggressive unmutated chronic lymphocytic leukaemias constitute two variants of the same disease. The reasons

<table>
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<th>Karyotype</th>
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<th>13q deletion</th>
<th>Trisomy 12</th>
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<td>Total patients (%)</td>
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<td>55</td>
<td>16</td>
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<td>53</td>
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<td>30</td>
<td>20</td>
<td>34</td>
<td>50</td>
<td>41</td>
</tr>
<tr>
<td>C</td>
<td>17</td>
<td>8</td>
<td>15</td>
<td>25</td>
<td>36</td>
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<td>Overall survival (months)</td>
<td>120</td>
<td>132</td>
<td>120</td>
<td>84</td>
<td>30</td>
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Table 1: Genetic aberrations in chronic lymphocytic leukaemia

*Data refer to frequency with which every cytogenetic profile is noted in the different Binet stages—ie, 18% of patients have a normal karyotype, of whom 53% are Binet stage A.
Defects in apoptosis and proliferation

Accumulation of mature B cells that have escaped programmed cell death and undergone cell-cycle arrest in the G0/G1 phase is the hallmark of chronic lymphocytic leukaemia.86 These cells have a low proliferative activity, and data lend support to the hypothesis that in-vivo defective apoptosis accounts for accumulation of B cells. In chronic lymphocytic leukaemia cells, translocations of the BCL2 gene are rare (fewer than 1% of cases)86 despite high amounts of the BCL2 protein. The role of BCL2 in apoptosis inhibition is not clear, since no correlation exists between in-vitro apoptosis and the amount of BCL2 expression.86 However, other members of the BCL2 family, such as anti-apoptotic proteins BCL-XL (alternative splice variant of BCL2L1), BAG1, and MCL1, are overexpressed whereas proapoptotic proteins, such as BAX and BCL-XS (alternative splice variant of BCL2L1), are underexpressed.86

Deregulation of cell-cycle regulatory genes might also contribute to accumulation of malignant cells in early phases (G0/G1) of the cell cycle. In chronic lymphocytic leukaemia cells, raised amounts of the cyclin negative regulator CDKN1B protein are recorded in most patients.85 In view of the key role of this protein in cell-cycle progression, its overexpression in chronic lymphocytic leukaemia cells could account for the accumulation of B cells in early phases of the cell cycle. These data suggest that chronic lymphocytic leukaemia is a disease resulting from accumulation rather than proliferation.

By contrast with in-vivo results, apoptosis happens after in-vitro culture, indicating a role of the microenvironment in chronic lymphocytic leukaemia cell survival.86,87 Findings showing that apoptosis in vitro is prevented by exposure to interleukin 4 and by stimulation via surface CD40 also favour this view. In vivo, such inhibition can happen in pseudo-follicles seen in the lymph nodes and in cell clusters described in bone marrow.88 These pseudo-follicles include, in close contact with proliferating B cells, increased numbers of CD4 T cells expressing CD40 ligand. These activated CD4 T cells could be recruited by tumour B cells, since they constitutively express the T-cell-attracting chemokines CCL17 and CCL22.88,89 This idea could be in agreement with a model of selective survival of certain clonal submembers, which would receive survival signals in these particular sites.

With a non-radioactive, stable isotopic labelling method to measure chronic lymphocytic leukaemia kinetics, Messmer and colleagues90 showed that B-cell chronic lymphocytic leukaemia is not a static disease, resulting simply from accumulation of long-lived lymphocytes, but is a disease with a dynamic process in which cells proliferate and die, sometimes at appreciable levels. This finding is in conflict with the view that chronic lymphocytic leukaemia is characterised almost exclusively by cell accumulation due to a defect in apoptosis. This mechanism might compensate for the clonal decrease that could take place in the periphery by apoptosis and, depending on its importance, could have a major role in regulation of tumour burden.

A picture is emerging that emphasises the importance of proliferation centres in lymph nodes spleen and bone marrow. Here, stimulation by CD31 on endothelial cells and CD154 on T-cells activates chronic lymphocytic leukaemia cells, upregulating CD38 and perhaps ZAP-70, provoking cell division and reinforcing resistance to apoptosis. From the proliferation centre the cell emerges into the circulation, where levels of activation markers begin to decline at a rate determined by intrinsic qualities of the cell. Cells that are better at retaining activation markers are drawn back into the tissues by chemokines and once there repeat the whole cycle. CD38 can be seen as an index of how recently the CLL cell has visited a proliferation center.93–94

Management

Assessment of prognosis

In chronic lymphocytic leukaemia, a third of patients never need treatment and have long survival; in another third, an initial indolent phase is followed by disease progression; the remaining third exhibit aggressive disease at onset and need immediate treatment.95 The Rai and Binet staging systems have enabled individuals with chronic lymphocytic leukaemia to be divided into three prognostic groups (good, intermediate, and poor) and have provided a foundation for clinicians to design therapeutic strategies for the disease. However, with neither the Rai nor the Binet staging system can we predict who in the good prognosis group will develop progressive disease.96 Several attempts have been made to address this deficiency. Lymphocyte doubling time, the pattern of bone-marrow involvement, and concentrations in serum of β2 microglobulin, thymidine kinase, and soluble CD23 all have some value but also important drawbacks.97 Lymphocyte counts can double in response to infection, vaccination, and steroid treatment; patterns of marrow involvement need invasive investigation; measurement of thymidine kinase in serum needs a radioassay; and amounts of CD23 and β2 microglobulin indicate both bulk of disease and rates of progression.

As reported above in the section on Genetic abnormalities, chronic lymphocytic leukaemias with mutated immunoglobulin genes have good prognosis and those with unmutated genes show poor prognosis.98,99 The mutational profile of immunoglobulin genes delineates prognostic groups within all Binet’s stages (table 2).100 The IGHV mutational profile has the advantage that it remains constant during clonal evolution, which contrasts with genomic aberrations and serum markers. Since sequencing IGHV genes is costly, time consuming, and unavailable at most medical facilities, detection of appropriate, reliable
surrogate markers for IGHV mutational status has attracted worldwide attention. An early candidate surrogate marker was expression of CD38. However, although CD38 expression is associated with poor prognosis, its relation to immunoglobulin mutational status remains controversial. Furthermore, expression of CD38 can change during disease evolution and concerns exist with respect to interlaboratory variations and the definition of the best cutoff value.

Crespo and colleagues developed a multivariable flow-cytometric test for ZAP70 that showed 95% correlation with IGHV gene mutational status; this finding was confirmed by a similar assay that used a slightly different way of expressing the results. However, these tests used indirect immunofluorescence, and a more convenient assay using direct immunofluorescence gave only 77% concordance with mutational status of IGHV genes. With the direct assay, ZAP70 seemed to be superior to IGHV genes in prediction of time-to-first treatment, whereas in ZAP70-negative patients, IGHV mutational status delineated good from intermediate prognosis. So far, this assay has not exported well to other laboratories and considerable dispute remains about how ZAP70 amounts should be estimated.

LPL is consistently overexpressed in patients with unmutated chronic lymphocytic leukaemia and has also been proposed as a surrogate marker. By contrast with ZAP70, which sometimes fails to identify advanced forms of disease, this marker seems to be an independent prognostic factor for individuals with Binet stage B and C disease. Assay by real-time quantitative PCR is less widely applicable than flow cytometry, but data suggest that this method is as good as IGHV mutational analysis and more reliable than ZAP70 as a prognostic factor.

For laboratories with facilities to measure concentrations in serum of thymidine kinase, high amounts at diagnosis identified patients categorised into good prognosis groups by other biomarkers (IGHV, ZAP70, CD38, del13q14) who subsequently progressed to advanced disease. Measurement of telomere length also refined the prognostic analysis of IGHV unmutated cases. Patients with short telomeres had significantly shorter progression-free and overall survival than did those with long telomeres. Low amounts of the chemokine receptor CXCR3 predict reduced survival independent of IGHV mutations and CD38 levels. The degree of upregulation of CLLU1 is an independent prognostic marker in patients younger than age 70 years.

Although all these markers provide useful prognostic information, the mutational status of IGHV genes and del17p and del11q are the most robust prognostic indicators, having been validated in prospective phase III clinical trials. Findings of a US Intergroup study comparing fludarabine with fludarabine plus cyclophosphamide showed that median progression-free survival was significantly lower for patients with del17p or del11q23 than for those with other cytogenetic findings. Although progression-free survival was longer for individuals with mutated IGHV genes than for those with unmutated genes, the study was insufficiently powered for this finding to reach significance. In the UK Leukaemia Research Fund CLL4 trial comparing fludarabine, fludarabine plus cyclophosphamide, and chlorambucil, the effect of prognostic markers on outcome was assessed prospectively. Importantly, prognostic factors had a greater effect on overall survival than did choice of treatment. Patients with del17p in more than 20% of cells had a significantly poorer response rate and median overall survival than did all other individuals, whereas those with unmutated IGHV genes or del11q23 had significantly shorter progression-free and overall survival than did those with mutated IGHV genes, no matter which treatment they were given. Findings of a CALGB 9712 phase II comparison of different schedules of rituximab given with fludarabine showed that median progression-free and overall survival were significantly greater in patients with mutated IGHV genes than in those with unmutated genes. Survival was also significantly increased with the Déhér hierarchical classification of FISH results moving from del17p13 to del13q14.

Combinations of prognostic factors might be more useful than individual factors. CD38 and IGHV mutations or CD38 and ZAP70 both perform better than any one factor. A scoring system based on six surface molecules (CD62L, SELE, CD54, ICAM1, CD49c, ITGA3, CD49d, ITGA4, CD38, and CD79B) detectable by flow cytometry has been proposed. Another using the easily available factors of age, sex, Rai stage, number of lymph nodes involved, absolute lymphocyte count, and β2 microglobulin has been assessed in many patients.

By multivariate analysis, both Binet staging and IGHV genes retain their independent prognostic significance in chronic lymphocytic leukaemia and are complementary. As table 2 shows, addition of Binet staging to the mutational profile of immunoglobulin genes and del17p deletion, which is the strongest independent prognostic marker, allows segregation of patients into five prognostic subgroups. We do not claim this prognostic system to be definitive; undoubtedly incorporation of other factors will

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<th>Patients (%)</th>
<th>Median survival</th>
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<td>Mutated stage A</td>
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<td>75% survival expectancy at 34 months</td>
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<td>Unmutated stage A</td>
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<td>97 months</td>
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<tr>
<td>Mutated stages B+C</td>
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<td>120 months</td>
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<tr>
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<td>78 months</td>
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<tr>
<td>17p deletion, any stage and mutational status</td>
<td>7</td>
<td>36 months</td>
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*Randomised clinical trials of early treatment versus wait and watch for early-stage patients with poor prognostic and mutational status 17p deletion, any stage

**Table 2: Prognostic classification of patients with chronic lymphocytic leukaemia by combination of Binet’s staging system with mutational status of IGHV genes and 17p deletion**
give greater refinement, but it makes use of the best established and validated factors.

In conclusion, recognition of novel biological variables has had a major effect on our understanding of chronic lymphocytic leukaemia. Some variables seem to be of considerable prognostic importance but, as yet, no evidence is available to suggest that changing therapeutic approaches on the basis of these results will lead to improvement in outcome. Prospective clinical trials are needed to address the stratification of patients according to these factors.

When to start treatment
Most patients with chronic lymphocytic leukaemia present without symptoms or signs; they are identified simply because a blood test has been requested for an unrelated reason. Many of these people never progress or need treatment; however, those who do need treatment usually present in the same way.

Findings of a meta-analysis of seven trials including 2048 early-stage patients randomly allocated either immediate or deferred treatment with chlorambucil (with or without prednisolone) showed no benefit for either treatment group. In a French study, 51% of Binet stage A patients allocated to the deferred group eventually needed treatment and 27% of this group died of a cause related to chronic lymphocytic leukaemia.

Standard management of chronic lymphocytic leukaemia, therefore, includes a period of watchful waiting until features of progression are noted. These signs—of bulk disease, lymphoma-related symptoms, and marrow failure—have been codified by a working group sponsored by the American National Cancer Institute. The document is currently under revision and is unlikely to include recommendations for changes in treatment based on new prognostic markers, but it is likely to recommend new randomised clinical trials with stratification based on such markers.

With more effective treatments than chlorambucil and better ways of establishing which patients are unlikely to progress, the strategy of watchful waiting should be revisited. Randomised trials of early versus delayed treatment for early-stage patients with poor-risk prognostic markers are planned or underway in Germany, France, the USA, and the UK. In a meta-analysis of ten trials including 2035 advanced-stage patients, addition of anthracycline or a vinca alkyloid to the alkylating agent was not shown to affect outcome.

Purine analogues
With the introduction of purine analogues, a class of drug better able to achieve complete remission in chronic lymphocytic leukaemia, the possibility has arisen of treating the disease in a similar way to other leukaemias—eg, with induction chemotherapy to achieve complete remission followed by consolidation treatment to eliminate minimal residual disease. Progress towards this end has been limited for several reasons. Complete remission in chronic lymphocytic leukaemia allows for up to 30% of chronic lymphocytic leukaemia cells to remain in the bone marrow; many patients live long and symptom-free lives without achieving complete remission; and most are elderly and are not candidates for more intensive treatments. Purine analogues cause profound suppression of T-cell immunity and can trigger autoimmunity. If the induction-consolidation strategy is not to be followed, these hazards could outweigh the benefits.

The purine analogue fludarabine, alone or in combination, has become the standard of care in most countries other than the UK. Researchers on a meta-analysis looked at five trials of 1838 patients randomly allocated either an alkylator-based regimen or a purine analogue. Individuals treated with purine analogues had significantly higher overall and complete response rates and longer progression-free survival than did those treated with alkylator-based regimens, but overall survival did not differ between treatment groups. Three further large trials had not been assessed at the time the meta-analysis was undertaken, and a difference in overall survival could yet arise as further data accumulate. However, because patients for whom one regimen fails can respond very well to another, this question might never be resolved. Because of this factor and because the natural history of chronic lymphocytic leukaemia is so long, researchers doing clinical trials have adopted progression-free survival as a surrogate for overall survival as the primary endpoint. However, such a strategy can be misleading.

Findings of a trial in which a high monthly dose of chlorambucil (70 mg/m²) was compared with fludarabine showed that response rates and median progression-free survival and overall survival did not differ between groups. Furthermore, fewer toxic effects arose in the chlorambucil group—ie, neutrophil counts lower than 1×10⁹/L, admission for more than 1 day, and grade 1 and 2 diarrhoea. The frequency of autoimmune haemolytic anaemia was similar in both groups but seems to have been more severe in the fludarabine group than in the chlorambucil group, since two patients treated with fludarabine died from the complication.

Combinations of purine analogues and alkylating agents have been tested in three randomised trials. In an Intergroup trial, a fludarabine plus chlorambucil regimen had to be abandoned as too toxic. Workers on a German CLL4 trial compared fludarabine plus cyclophosphamide with fludarabine alone as first-line therapy in 375 patients younger than age 66 years, and a similar comparison was undertaken in a British CLL4 trial in 390 patients without age restriction. Findings of both CLL4 studies showed the combination was significantly better than fludarabine alone in terms of overall and complete response rates and progression-free survival, but overall survival did not differ between groups. Moreover, the combination was significantly more toxic than fludarabine alone in terms of neutrophil counts lower than 1×10⁹/L, admission for more
than 1 day, grade 3 and 4 nausea and vomiting, grade 1 or 2 alopecia, and grade 1 or 2 diarrhoea. However, autoimmune haemolytic anaemia was significantly less frequent with the combination. In Intergroup trial E2997, complete response rates were reported as 24–6% for fludarabine plus cyclophosphamide and 5–3% for fludarabine alone. Median progression-free survival was 33·5 months and 19·9 months, respectively. Both these differences were statistically significant.

Another purge analogue, cladribine, has been assessed in a three-way, randomised phase III study. Researchers compared the agent alone and in combination with cyclophosphamide or cyclophosphamide plus mitoxantrone. The three-drug combination produced significantly more responses and complete remissions at the expense of more bone-marrow toxic effects. Progression-free and overall survival did not differ between groups. Mitoxantrone has also been used in combination with fludarabine plus cyclophosphamide in a phase II trial in relapsed or resistant chronic lymphocytic leukaemia. The findings of this trial were remarkable because the complete remission rate was 50%, with a third of these patients having no detectable disease with a very sensitive method.

**Immunotherapy**

Rituximab—the chimeric monoclonal anti-CD20—is only moderately active as a first-line agent in chronic lymphocytic leukaemia, with an overall response rate of 51% and a complete remission rate of only 4%. However, when it is added to fludarabine or fludarabine plus cyclophosphamide, impressive responses have been reported. In a randomised phase II study, rituximab added to fludarabine produced higher responses when given concurrently than when given sequentially. When compared with historical controls and in a multivariate analysis controlling for pretreatment characteristics (but not for modern prognostic markers), addition of rituximab seemed to enhance significantly progression-free and overall survival.

The combination of fludarabine, cyclophosphamide, and rituximab has been tested in first-line and relapsed and refractory settings (see also section on Drug-resistant chronic lymphocytic leukaemia). As first-line therapy, overall response rates of 95% and complete remission rates of 70% were reported. In historical controls treated with fludarabine plus cyclophosphamide, the same research group recorded overall response rates of 88%, with 35% complete remission. However, only 33% of patients treated with fludarabine, cyclophosphamide, and rituximab were Rai stage III and IV, compared with 50% of those given fludarabine plus cyclophosphamide, and no modern prognostic markers have been reported for either patients or historical controls. In another phase II study, addition of mitoxantrone to fludarabine, cyclophosphamide, and rituximab seemed to add only toxic effects rather than increased efficacy. Phase III comparisons of fludarabine, cyclophosphamide, and rituximab and fludarabine plus cyclophosphamide are currently underway, and these findings should be reported in 2008 or 2009.

Another purge analogue, pentostatin, has been assessed in combination with cyclophosphamide and rituximab in a phase II trial of previously untreated patients with chronic lymphocytic leukaemia. This trial is valuable in that the prognostic markers IGHV gene mutations, CD38 expression, ZAP70 expression, and interphase cytogenetics were reported. The overall response rate was 91%, with 41% complete remission. Patients with TP53 anomalies had poor responses. The researchers claim that this regimen is less toxic than fludarabine, cyclophosphamide, and rituximab.

Alemtuzumab seems to be one of the few agents capable of killing chronic lymphocytic leukaemia cells with mutated or deleted TP53 genes. The drug has been used as first-line therapy in a phase III trial, in which it was compared with chlorambucil at a dose of 40 mg/m² per month. Overall response rates and progression-free survival were significantly better for alemtuzumab than for chlorambucil.

**Consolidation therapy**

Consolidation of remission aims to eliminate all detectable disease from the patient. In assessing the success of such attempts, the sensitivity of the method used to detect minimal residual disease is crucial. Unlike, many other haematological cancers, there is no characteristic chromosomal translocation to detect. Detection methods use either PCR to identify a unique tumour-associated sequence or flow cytometry to find an exclusive set of tumour antigens. The most sensitive assay uses PCR to detect a specific clonotypic sequence. This technique can ascertain 1 in 100 000 cells, but the IGHV gene needs to have been sequenced before treatment. Use of consensus primers to detect monoclonal immunoglobulin is much less sensitive, being only able to identify 1 in 1000 cells. Rawstron and colleagues have developed a four-colour flow technique capable of detecting 1 in 50 000 cells, and this method has been refined further by an international group for use when anti-CD20 form part of the treatment strategy.

Remissions are traditionally consolidated with high-dose chemotherapy, sometimes with autologous stem-cell rescue. With this approach, a German research group could eliminate minimal residual disease and produce prolonged remissions in patients whose chronic lymphocytic leukaemia had mutated IGHV genes; however, for those with unmutated IGHV genes, molecular relapse was inevitable, and clinical relapse almost so, by 4 years’ follow up. However, even this less-than-encouraging outcome might be better than what is achievable with conventional chemotherapy, according to findings of a comparison in matched historical controls. Moreover, stem-cell harvest is only possible in about two-thirds of patients with chronic lymphocytic leukaemia, and there is a worryingly high prevalence of secondary myelodysplastic syndrome.
Stem-cell allografts have been used in an attempt to capitalise on the graft-versus-leukaemia effect to eliminate minimal residual disease. In a largely elderly population, myeloablative conditioning leads to high treatment-related mortality of 40–50%. To combat this toxic effect, allotransplantation with reduced intensity conditioning, often followed by donor lymphocyte infusion, has become popular. Early results do not yet show an improvement in event-free survival compared with myeloablative transplants. In every series, a high frequency of chronic graft-versus-host disease is noted. Nevertheless, in patients with poor-risk prognostic markers, and especially those with 17p13 deletions, sustained remissions without molecular relapse are achievable.

Potentially less hazardous are attempts to consolidate remission with monoclonal antibodies. Alemtuzumab is especially suited because it can kill chronic lymphocytic leukaemia cells in a caspase-independent manner that is not inhibited by loss of TP53 protein and is, therefore, able to eliminate chemotherapy-resistant cells. In a study of 91 previously treated patients, 44 of whom were refractory to purine analogues, complete remissions as defined by the US National Cancer Institute were achieved in 32%. In these individuals, a four-colour flow technique for detection of minimal residual disease did not identify disease in 56%. Patients who became negative for minimal residual disease had significantly longer treatment-free and overall survival than those who did not.

In a prospective phase III study in Germany, patients were randomly allocated either no treatment or alemtuzumab 30 mg intravenously three times a week for 12 weeks, beginning a median of 67 days after the last dose of induction chemotherapy (fludarabine with or without cyclophosphamide). Although recruitment to this trial was halted at 21 patients because of severe infections in the alemtuzumab group, those allocated alemtuzumab had significantly longer progression-free survival than those assigned no treatment. Using a very sensitive PCR-based assay with sequence-specific primers to detect minimal residual disease, five of six patients tested became negative for minimal residual disease. Findings of a phase II study of alemtuzumab given in doses of 10 mg subcutaneously three times a week for 6 weeks, beginning not less than 8 weeks after discontinuation of fludarabine induction chemotherapy, proved that this regimen was safe and able to turn partial remissions into complete remissions. A PCR technique with consensus primers (a less sensitive method than that used in the phase III study) was used to detect minimal residual disease; patients with mutated IGHV genes were more likely to become negative for minimal residual disease than were those with unmutated IGHV genes. Reactivation of cytomegalovirus happened in 53% but was successfully treated with oral ganciclovir. 53% of patients successfully underwent autologous stem-cell transplantation, but no follow-up information is available.

At present, immunological consolidation with alemtuzumab after chemotherapy seems safer than with allogeneic transplantation, but we await a comparison of efficacy.

**Drug-resistant chronic lymphocytic leukaemia**

After successful induction therapy, relapse is almost inevitable if no consolidation takes place and is still possible if consolidation has been done. After long remissions, patients will usually respond again to the same type of treatment, but relapse after short-lived remissions and primary refractory disease needs a different approach.

In the relapsed and refractory setting, fludarabine, cyclophosphamide, and rituximab can produce high response rates (73%) and complete remissions (25%). In a comparison with historical controls, results suggested that fludarabine, cyclophosphamide, and rituximab is superior to fludarabine plus cyclophosphamide or to fludarabine alone. However, although the multivariate analysis included concentrations in serum of $\beta$2 microglobulin, interphase cytogenetics and IGHV mutational status were not studied.

Most effective treatments for chronic lymphocytic leukaemia need an intact TP53 pathway. Patients with advanced refractory chronic lymphocytic leukaemia treated with high-dose prednisolone had an overall response rate of 77%. Alemtuzumab also produced high levels of response in TP53-deleted chronic lymphocytic leukaemia. The combination of these agents gave a 100% response with 60% complete remission in a few patients with TP53 defects.

Flavopiridol, a cyclin-dependent kinase inhibitor, has proved a very effective killer of TP53-deleted chronic lymphocytic leukaemia cells in vitro, but it was almost completely ineffective in vivo because it was so highly bound to human serum albumin. By altering the infusion schedule, partial remissions of long duration have been obtained in 42% of chronic lymphocytic leukaemias with TP53 defects.

**New agents**

A whole range of new therapeutic agents is in development, some of which are already in the clinic. Lenalidomide might act by interfering with the tumour/stroma interaction. Ofatumumab is a fully humanised CD20 monoclonal antibody with reputed advantages over rituximab because of a slower off-rate. Lumiliximab—a primatised anti-CD23—is entering clinical trials in patients with chronic lymphocytic leukaemia. Chronic lymphocytic leukaemia cells with deletions at 11q23 seem to be especially sensitive to the orally available poly (ADP-ribose) polymerase inhibitor 4-amino-1,8-naphthalamidine. ATM-deletion-mediated drug resistance might also be overcome with Nutlin 3a.

Finally, acadesine seems to be a new chemotherapeutic agent capable of killing chronic lymphocytic leukaemia cells yet leaving T cells unharmed.
Conclusions

Only a few patients with chronic lymphocytic leukaemia are likely to be eligible for an induction-consolidation strategy. For most individuals, further clinical trials are needed that make use of modern prognostic markers to stratify patients into treatment groups.

Conflict of interest statement

TJH is currently chairman of the data and safety monitoring board for the REACH clinical trial, in which previously untreated patients with stages B and C chronic lymphocytic leukaemia are randomly allocated fludarabine and cyclophosphamide either with or without rituximab, and receives a fee from Roche Pharmaceuticals for every meeting chaired. TJH is also supported by a grant from Tenovus. GD is currently a member of the data and safety monitoring boards for the REACH clinical trial and for the CLL8 trial, in which previously untreated patients with stages B and C chronic lymphocytic leukaemia are randomly allocated fludarabine and cyclophosphamide either with or without rituximab, and receives a fee from Roche Pharmaceuticals for every meeting attended.

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Seminar


