Background and Objectives. B-cell chronic lymphocytic leukemia (B-CLL) results from the accumulation of monoclonal CD5⁺ B cells. Despite its homogeneity at cellular level, B-CLL is clinically heterogeneous. Clinical studies indicate that CD38⁺ B-CLL are characterized by a more aggressive clinical course than are CD38⁻ B-CLL. On the basis of these studies and considering the established correlation between specific chromosome aberrations and the clinical course of B-CLL, it is possible that CD38⁺ B-CLL cases are also characterized by specific subsets of chromosome alterations.

Results. We found a clear correlation between the presence of chromosomal imbalances and CD38 expression: 13/16 CD38⁺ cases had chromosome imbalances, most of them (12/13) correlated with a poor prognosis. Among the CD38⁺ B-CLL patients, only 8/36 displayed chromosome imbalances; the only three cases with loss in 13q as a single aberration, considered a good prognostic marker, were in this group. Moreover, we found that cytogenetic alterations were also more complex in the CD38⁺ B-CLL subset, since 9/10 with two or more aberrations were in the CD38⁺ group.

Interpretation and Conclusions. Collectively, the data reinforce the value of CD38 as a prognostic factor and indicate that genotypic/phenotypic features distinguish B-CLL subsets.

Key words: B-CLL, molecular cytogenetics, chromosome aberrations, immunophenotype, CD38.

B-cell chronic lymphocytic leukemia (B-CLL) results from the progressive accumulation of monoclonal CD5-positive B cells. Despite its homogeneity at cellular level, B-CLL is clinically heterogeneous since some patients survive for a long time without therapy, while others progress towards more advanced stages and die despite aggressive treatment.¹

These clinical observations have prompted numerous studies aimed at determining reliable prognostic markers capable of predicting the progression and outcome of the disease from its early stages. Following the initial attempts which failed to detect reliable predictive markers, the advent of cellular and molecular biology methodologies has made it possible to determine that certain biological features of the neoplastic cells may be strictly connected with their aggressive behavior in vivo.²⁻³ For example, B-CLL that utilize mutated V₅/Ig genes have a better clinical outcome than those with unmutated genes.²⁻⁵ B-CLL can also be subdivided into two groups by CD38 staining, i.e. a group whose malignant cells express high levels of CD38 and another group with low CD38 expression. CD38⁺ and CD38⁻ B-CLL cells differ in a number of functional features including propensity to spontaneous apoptosis in vitro and capacity to respond to signals delivered through the B-cell receptor (BCR).⁶⁻⁷ Moreover, a number of clinical studies have found that CD38⁺ B-CLL have a more aggressive clinical behavior than do the CD38⁻ B-CLL, although these data have not been confirmed by other investigators.⁸⁻¹⁸ Conventional cytogenetic studies reported that half of B-CLL cases examined showed no chromosomal aberration.¹⁹ Indeed B-CLL cells exhibit low proliferative activity and are very difficult to stimulate, even using B-cell specific mitogens. Moreover metaphases may arise from non-leukemic T-lymphocytes. In the last decade, molecular techniques have been applied to B-CLL to detect chromosomal aberrations in interphase nuclei.²⁰⁻²² Interphase fluorescence in situ hybridization (FISH) revealed the presence of cell clones carrying chromosomal aberrations in cases in which no aberrations had been detected by classical cytogenetics.²³ Using this technique on a cohort of 325 B-CLL patients, Döhner²⁴ showed that the overall frequency of patients with chromosomal aberrations rose above 80%. In this study, deletion in 13q was the most frequent aberration (55%) followed by deletion in 11q (18%), while these aberrations were almost inexistent by banding technique.²⁴⁻²⁵ Using FISH, a number of investigators have determined the most frequent alterations in B-CLL,²³ and also drawn
some correlation between their presence and the clinical course of the disease. However, FISH depends on prior knowledge of the specific aberrations to be screened for. This limitation does not apply to comparative genomic hybridization (CGH), a molecular cytogenetic method that allows indirect screening for copy number aberrations throughout the genome in a single hybridization. CGH is essential in cytogenetic studies of solid tumors, but also has potential clinical use in the diagnosis of recurrent chromosome aberrations in certain leukemias and lymphomas. Indeed, studies performed on B-CLL patients using CGH together with classical cytogenetics and interphase FISH confirmed the ability of CGH to identify chromosome imbalances. It is noteworthy that, in a significant number of cases, CGH and interphase FISH reveal chromosome imbalances not detected by banding analysis, possibly because the malignant clone is not present in the proliferating cell fraction. CGH is not able to detect structural aberrations without loss or gain of chromosomal material; this is not, however, a major limitation in B-CLL, in which translocations are poorly represented.

Recently, a correlation between the presence of chromosomal abnormalities with known negative prognostic value and CD38 expression has been suggested. In the present work, we wanted to ascertain whether chromosomal abnormalities detected by CGH showed any correlation with CD38 expression.

### Design and Methods

#### Clinical features of patients

Fifty-two patients with B-CLL (32 males and 20 females) were studied. Their characteristics are described in Table 1. The patients were numbered when the analyses were performed, irrespectively of their presentation. Only 8 patients were examined at diagnosis. The mean age at diagnosis was 60 years (median, 61; range, 36-81). The male to female ratio was 1.6:1. Patients were staged at diagnosis according to the Rai and Binet classification. Eighty-five percent of patients had Rai stage 0, I or II disease, and 89% of patients had Binet stage A or B disease. Thirty-one patients had received a chemotherapeutic regimen before CGH and CD38 analysis. Twenty-three patients were examined at diagnosis.

#### Cell preparation, fluorescent staining and flow cytometry analyses

Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood by density gradient centrifugation using Ficoll-Hypaque (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). PBMC were cryopreserved and thawed at the time of analysis. B cells were purified from PBMC by removing monocytes and CD3+ T cells by adherence to plastic surfaces and magnetic beads, respectively. Flow cytometry analysis was performed to analyze the purified B-CLL cells. The following conjugated antibodies were used in double or single immunofluorescence: FITC-CD3/PE-DR, PE-CD5/FITC-CD19, PE-CD23/FITC-CD22, PE-CD10, Fluorescein-conjugated IgM, PE-conjugated anti-CD3, and biotin-conjugated anti-CD5.
from Becton Dickinson (San José, CA, USA), FITC-IgM, FITC-IgD, FITC-CD68 from Dakopatts (Glostrup, Denmark) and FITC-CD44 from Pharmigen. Stained cells were analyzed on a FACS sort (Becton Dickinson Immunocytometry Systems).

The Cellquest Software System was used to acquire and to analyze data. This phenotypic analysis showed that purified B-CLL cells from all the 52 cases comprised more than 95% of double positive CD5+/CD19+ cells and less than 5% of CD3+ or CD68+ cells. The cells were also CD23+, CD44 bright, CD22 dim/negative, CD10+, IgM and IgD dim/negative. CD38 expression on purified B-CLL cells was studied using double immunofluorescence FITC-CD19/PE-CD38. B-CLL cases were considered CD38+ when the percentage of CD38+ cells exceeded 30%.

CGH

To increase the sensitivity of the CGH, B cells were purified from PBMC by removing monocytes and CD3+ T cells by adherence to plastic surfaces and magnetic beads, respectively. DNA extraction and CGH were performed as described elsewhere. Briefly, normal human genomic DNA (control DNA) was labeled with digoxigenin-11-deoxyuridine triphosphate (dig-11-dUTP, Roche Diagnostics, Milan, Italy), and B-CLL cell DNA (test DNA) was labeled with biotin-16-deoxyuridine triphosphate (bio-16-dUTP, Roche Diagnostics) by a standard nick translation reaction. The concentration of DNase I was adjusted to result in an average fragment size of 200 to 1500 bp. Then, 250 ng of labeled test DNA, 250 ng of labeled control DNA, and 30 µg of human Cot-1 DNA (GIBCO-BRL-Life Technologies) were co-hybridized to slides with metaphases prepared from the blood of a healthy, male donor. After hybridization for 3 days and post-hybridization washes, test and control DNA were detected with fluoresceinated (FITC)-avidin (Vector, Burlingame, USA) and anti-dig rhodamine (TRITC)-conjugated antibody, respectively (Roche Diagnostics). Chromosomes were counterstained with 4,6-diamidino-2-phenylindole (DAPI), resulting in a Q banding-like pattern that was used for chromosome identification.

Digital image analysis

Image acquisition, processing, and evaluation were performed as described elsewhere. Images were acquired using an epifluorescence microscope (Provis AX70, Olympus, Italy) equipped with a digital CCD camera (C4742 Orca II, Hamamatsu, Japan). DAPI, FITC and TRITC images of metaphase cells were acquired with selective single-bandpass filters. Ratio profiles of individual chromosomes were obtained with dedicated software (Cromodigit, Casti Imaging, Italy). For each experiment, the mean ratio profiles of ten metaphase cells were calculated. Thresholds for the identification of imbalances were defined as 0.75 (lower threshold) and 1.25 (upper threshold). Chromosomes or chromosome regions with a fluorescence ratio outside this interval were considered to be over- or under-represented, respectively.

Results

Identification of two groups of B-CLL according to CD38 expression

The purified malignant B-CLL cells from 52 patients were double stained with CD38 and CD19 monoclonal antibodies. Two groups of B-CLL, i.e. CD38+ and CD38− B-CLL, were identified using the cut-off limit of 30%, already utilized in previous studies. The typical flow cytometry profiles observed in one CD38+ and in one CD38− case are shown in Figure 1a, while the results of all the cases are summarized in Figure 1b. The two groups of patients did not differ significantly for age, sex or Rai/Binet staging using Fisher’s exact test or Cox analysis (data not shown).
CGH analysis

Chromosome imbalances were detected by CGH. Two examples of merged CGH images and the relative mean profiles of ten metaphases with chromosomal imbalances are shown in Figure 2. Twenty-one out 52 patients (40%) showed chromosome imbalances (Table 1). Of these, 11 had a single aberration (21.2%), 8 showed 2 aberrations (15.4%) and 2 had a complex karyotype (3.8%). The most frequent aberration was gain of chromosome 12 (9 cases), followed by a loss in 11q (5 cases), a loss in 13q (4 cases), gain of chromosome 18 (always associated with a gain of chromosome 12) (3 cases), the loss of X-chromosome (3 cases), and a loss in 6q (2 cases). The following aberrations were detected only once: loss in 3p, 4p, 8p and 17p, loss of chromosome 16, gain of chromosomes 7 and gain in chromosome 3q. The complete CGH data are listed in Table 1 and summarized in Figure 3. The patients in Table 1 were subdivided into two groups according to CD38 expression. When CD38+ and CD38− B-CLL cases were considered separately, it
was found that only 3/31 cases without aberrations were CD38+ (Table 1 and Figure 4). Among the 21 samples with chromosome imbalances, 13 were CD38+: 4 of them showed a single aberration (in 2 cases a trisomy of chromosome 12, in 1 case a loss in 11q and in 1 case a gain in 3q). Furthermore, all but one of the cases with more than one chromosome imbalance were detected in CD38+ patients (9 out of 10). Seven out 8 cases with chromosome imbalance and a low percentage of CD38 cells showed a single aberration (in 3 cases a loss in 13q, in 2 cases a loss of X chromosome, in 1 case gain of chromosome 12 and in 1 case a loss in 11q) while 1 case showed losses in 3p and 8p. When the percentages of CD38+ B-CLL cells in the groups without and with chromosome imbalances were compared, statistically significant differences were found (p=0.0001, two-sided Fisher’s exact test; Table 2).

### Discussion

Samples from 52 patients with typical B-CLL, diagnosed according to morphology and surface phenotype, were subjected to CGH analysis in order to obtain a comprehensive view of chromosomal gains and losses and to identify copy number aberrations specific for this pathology. To increase the sensitivity of CGH, we purified B-cells from the peripheral blood of B-CLL patients when there were less than 90% B cells. Twenty-one out of 52 (40%)
patients showed chromosome imbalances; 11/21 had single imbalances, whereas the remaining 10 patients had two or more chromosome alterations. Thirty-three per cent of patients had received chemotherapy before cytogenetic analysis. The presence of patients subjected to therapy in the cohort can hardly be avoided in this kind of study. As an example, a recent study based on a large cohort of patients included a similar percentage of patients with chromosomal abnormalities and CD38 expression by the malignant cells. Thirteen out of 16 CD38+ patients had chromosomal abnormalities, whereas, among the 8 CD38− patients only 2 displayed chromosome imbalances. These differences are highly significant (p=0.0001). Three out of the 8 CD38− patients with chromosomal alterations had a loss in 13q as a single aberration, which generally correlates with a good prognosis, 3 patients had rare alterations, the prognostic value of which remains to be determined, while the remaining 2 patients had aberrations correlated with a poor prognosis (−11q; +12). Twelve of 13 CD38+ patients with chromosomal alterations displayed aberrations that are correlated with a poor clinical outcome (−11q; +12; −17p), whereas one patient had a gain in

Figure 4. Percentages of CD38+/CD19+ purified B-CLL cells among cases without and with chromosome imbalances. ◇ cases without imbalances; △ cases with -13q as the sole imbalance; □ cases with chromosome imbalances of unknown prognostic significance only; ○ cases with chromosome imbalances with poor prognostic significance.

The B-CLL cases in this study could be subdivided into two groups according to the surface expression of CD38 by the malignant cells. This confirms previous findings from our laboratory and is also in line with data reported by others. The notion that CD38 staining is capable of distinguishing B-CLL subsets is not, however, accepted by all investigators, since some are apparently unable to distinguish B-CLL subsets based on the percentage or intensity of staining of the malignant cells with CD38 monoclonal antibodies. The reasons for these discrepancies, which have generated considerable debate, are not readily apparent and the issue is made even more complicated by differences in the reagents and the staining procedures used.

The striking finding of this study was the clear correlation existing between the presence of chromosomal abnormalities and CD38 expression by the malignant cells. Thirteen out of 16 CD38+ patients also had chromosomal abnormalities, whereas, among the 36 CD38− patients only 8 displayed chromosomal imbalances. These differences are highly significant (p=0.0001). Three out of the 8 CD38− patients with chromosomal alterations had a loss in 13q as a single aberration, which generally correlates with a good prognosis, 3 patients had rare alterations, the prognostic value of which remains to be determined, while the remaining 2 patients had aberrations correlated with a poor prognosis (−11q; +12). Twelve of 13 CD38+ patients with chromosomal alterations displayed aberrations that are correlated with a poor clinical outcome (−11q; +12; −17p), whereas one patient had a gain in

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chromosome 3q, which is rarely found in B-CLL and is hence of undetermined prognostic value. Moreover, of the 10 patients with two or more chromosome imbalances (another marker of poor prognosis) detected in this study, 9 were within the CD38+ group. Remarkably, among the cases with the highest values of CD38 expression, 3 cases had simultaneous gain of chromosomes 12 and 18. Gain of chromosome 18 never appeared alone, but was always associated with gain of chromosome 12. Although this association has already been described in B-CLL by classical cytogenetic studies, its significance and real frequency are not well documented. This is, in part, because most of the studies on chromosomal aberrations in large cohorts of B-CLL patients were performed with FISH using a panel of probes not including the chromosome 18 probe.

Our data are in keeping with the recent observations that the unbalanced distribution of genomic aberrations in IgVh high mutation and low mutation subgroups might point toward a distinct biological background in such B-CLL subgroups and may in part, explain their different behaviors. In the study by Kröber et al., genomic aberrations and Vh mutation status appeared to have a complementary role in estimating prognosis. Although CD38 expression has been proposed as an easily performed surrogate of Vh mutational status analysis, its prognostic value is not completely clarified. Moreover, the relationship between CD38 expression and chromosomal aberrations has not been extensively studied. In a recent paper, Chevallier et al. studied the prognostic value of CD38 expression and chromosomal abnormalities in a cohort of 125 patients (98 with typical and 21 with atypical morphology). They found the 11q deletion to be the only alteration associated with CD38+ phenotype. They also observed a significant correlation of trisomy 12 with progression, but not with survival. Oscier et al. showed that advanced stage, male sex, atypical morphology, CD38 expression higher than 30%, trisomy 12, 11q deletion, loss or mutation of p53 gene and unmutated IgVh genes were all poor prognostic factors in a univariate analysis, but only loss or mutation of p53 gene and unmutated IgVh genes retained prognostic significance in a multivariate analysis. Surprisingly, even the 11q deletion did not emerge as an independent prognostic factor.

Concerning the prognostic significance of CD38 expression in multivariate analysis, the authors suggested that a much larger group of patients was needed. Oscier et al. showed that the mean survival of patients with loss in 17p was the shortest (47 months). In the present study the groups of patients with and without chromosomal alterations do not differ in terms of survival (not shown) probably since all but 2 patients are still alive. However, the simple patient in our cohort with loss in 17p at diagnosis was followed for only 1 year and was experiencing a poor clinical course.

The different biological properties showed by B-CLL cells, including the expression of CD38, can help to explain the differences in the patients’ outcomes. Recent studies, including those from our laboratory, demonstrated that CD38- B-CLL cells with unmutated Vh/Vb region genes have a viable IgM initiated signal transduction pathway. This pathway can lead to proliferation/differentiation or apoptosis depending on co-signals received by the cells in vitro. In contrast, most of the CD38+ mutated B-CLL cells do not respond to signals delivered to surface Ig. Therefore, the interaction between the cells and the environment via B-cell receptor is much less marked in CD38+ mutated cases than in CD38- unmutated cases. These data suggest that CD38+ B-CLL cells are likely to be continuously stimulated via surface Ig.

This is related to the fact that surface Ig, encoded by unmutated Vh/Vb genes, retain natural antibody activity and hence can react continuously with autoantigens in vivo. In the case of surface CD38- negative, mutated B-CLL cells, it is unlikely that the B-cell receptor can exert a promoting role on cell expansion since there is not a viable IgM signal transducing pathway. Moreover, Ig encoded by mutated Vh/Vb genes rarely have natural antibody activity and, therefore, can rarely encounter the appropriate foreign antigen. Collectively, these considerations raise the issue of whether antigenic stimulation in B-CLL continues to exert a promoting effect on the growth of the malignant cells following transformation, and whether this is the reason for the clinical differences in B-CLL. Finally, it is unlikely that CD38 is solely a marker of cellular differentiation and clinical course. It is more probable that it also functions as a signaling molecule and, therefore may be directly involved in differences in disease severity. CD38 is known to play a role as an accessory molecule in B-cell receptor mediated signal transduction, as well as regulating cell apoptosis in certain normal B-cell subsets. Further studies will be necessary to clarify this topic.

A number of conclusions can be drawn from this study. First, considering the increasing recognition of the importance of chromosome alterations in predicting the clinical outcome of B-CLL, the observation that chromosomal alterations are significantly more frequent within the CD38-positive cases lends further support to the prognostic value of the surface marker, CD38. Second, the finding that CD38+ cases can be subdivided into two groups (i.e. with and without chromosomal alterations) may lead to the delineation of further prognostic subsets of B-CLL. Third, the paucity of chromosomal alter-
ations observed in the CD38− B-CLL may indicate that this group is more homogeneous, although a multiple marker study may help to disentangle a subset of cases characterized by special clinical features also in this category.

References


Pre-publication Report & Outcomes of Peer Review

Contributions
LO, SV, AZ performed the CGH studies and wrote the paper; SZ performed the immunophenotyping analysis; ER and MS performed clinical studies of patients; AA and MF were responsible for the study design. All the authors participated in the conception of the study and the interpretation of the data, and all critically revised the article. Primary responsibility for the paper, Tables 1 and 2 and Figures 2-4: LO; primary responsibility for Figure 1: SZ.

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In the following paragraphs, Dr. Pettitt summarizes the peer-review process and its outcomes.

What is already known on this topic
Many cell biological factors have now been identified in CLL that predict for adverse clinical outcome. Among these, the most important are relative lack of VH gene mutation, expression of ZAP-70, abnormalities of the p53 pathway, certain chromosomal abnormalities (17p13 deletion, 11q23 deletion and trisomy 12) and, somewhat controversially, CD38 expression. Associations have previously been reported between unmutated VH genes and CD38 expression, and between unmutated VH genes and adverse karyotype. However, these associations are imperfect, and there is relatively little existing information concerning the relationship between CD38 expression and karyotypic abnormalities.

What this study adds
Using the technique of comparative genomic hybridisation, the present study helps to establish this missing link by demonstrating a much higher frequency of adverse chromosomal aberrations (predominantly 11q23 deletion and trisomy 12) among the one third of patients whose tumor cells express CD38 (using a 30% cut-off value). This important new observation adds weight to the notion that CD38 expression really is an adverse prognostic factor in CLL, and reinforces the idea that unmutated VH genes, CD38 expression and adverse karyotype are mutually associated with one another.