Leukemia-cell proliferation and disease progression in patients with early stage chronic lymphocytic leukemia

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Abstract

The clinical course of patients with recently diagnosed early stage chronic lymphocytic leukemia (CLL) is highly variable. We examined the relationship between CLL-cell birth rate and treatment-free survival (TFS) in 97 patients with recently diagnosed, Rai stage 0–II CLL in a blinded, prospective study, using *in vivo* ^2H_2O labeling. Birth rates ranged from 0.07–1.31% new cells per day. With median follow-up of 4.0 years, 33 subjects (34%) required treatment by NCI criteria. High birth rate was observed in 44% of subjects and was significantly associated with shorter TFS, unmutated *IGHV* status, and expression of ZAP70 and of CD38. In multivariable modeling considering age, gender, Rai stage, expression of ZAP70 or CD38, *IGHV* mutation status and FISH cytogenetics, only CLL-cell birth rate and *IGHV* mutation status met criteria for inclusion.

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Hazard ratios were 3.51 (p=0.002) for high birth rate and 4.93 (p<0.001) for unmutated *IGHV*. The association between elevated birth rate and shorter TFS was observed in subjects with either mutated or unmutated *IGHV*s, and the use of both markers was a better predictor of TFS than either parameter alone. Thus, an increased CLL birth rate in early stage disease is a strong predictor of disease progression and earlier treatment.

INTRODUCTION

Chronic lymphocytic leukemia (CLL), the most frequent adult leukemia in the Western world, is notable for its highly variable clinical course. Some patients may progress early, while others may have an indolent course and not require therapy for years, if at all.[1, 2] Current guidelines do not recommend therapy for patients with recently diagnosed early stage CLL unless they demonstrate disease-related symptoms or complications or clear evidence of progressive disease. An ongoing clinical challenge is to identify patients who will develop aggressive disease and thus potentially might benefit from earlier treatment.

The staging systems of Rai[3] and Binet[4] remain the mainstays for classifying patient prognosis and survival. However, both systems have limited ability to predict early disease-progression.[5] With an increasing array of treatment options for CLL[6, 7] and with the majority of patients diagnosed with early stage disease, it is imperative to better understand the factors that put patients at highest risk for disease progression.

Prognostic markers based on CLL biology, including CLL-cell expression of ZAP70[8] CD38[9] and CD49d[10], leukemia-cell chromosomal aberrations[11], and *IGHV* mutational status[9, 12] have shown promise in early stage CLL and have advanced our understanding of the disease process.

In CLL, as with any malignancy, disease progression represents an imbalance between cell birth and death, ultimately leading to an accumulation of leukemia cells. Measures of the rates of *in vivo* CLL cell birth (proliferation) and disappearance from blood (true cell death plus recirculation to solid tissues) have provided important insights into the pathophysiology of the disease.[13–15] Although the classic view of CLL involved failed cell death in the setting of minimal cell proliferation,[16] data from several small studies demonstrate that some patients with CLL can have birth rates greater than those seen for normal B-cells.[13–15] Moreover, in a cross-sectional study that included patients with both early and late stage CLL, cell birth rates above 0.35% per day were associated with more aggressive disease, even in the setting of a low lymphocyte doubling time.[13] An unanswered question that is addressed in this study is the extent to which an increased birth rate in early stage CLL associates with more rapid disease progression and shorter treatment free survival (TFS).

MATERIALS AND METHODS

The study protocol was approved by the institutional review boards of all the participating clinical sites as well as by Western IRB.

Patients

Between 2005 and 2009, 119 subjects with CLL provided written informed consent, were enrolled at CLL Research Consortium (CRC) institutions and had CLL cell kinetics measured using orally administered ^2H_2O. Eligibility criteria included age > 18 years, CLL diagnosis within 3 years of enrollment, and early stage disease (Rai stage 0, I, or II). Patients with current or prior CLL treatment or those anticipated to need treatment within 16 weeks after enrollment were excluded, as were patients with serious co-morbid medical conditions and pregnant women.

Treatment Free Survival (TFS)

Clinical follow up information available through November 2013 was used for analysis. TFS was defined as the time from enrollment and initiation of ^2H_2O (deuterated or "heavy water") ingestion to the initiation of therapy according to the NCI criteria.[17] Patients who were not treated were censored at the time they were last known to remain untreated. Patients who enrolled in intervention studies testing strategies to delay initial treatment were censored at the time of enrollment in the intervention study. Patients were to be followed for a minimum of 2 years from the end of ^2H_2O ingestion. The study was blinded with cellular kinetics and clinical outcomes linked only after all data were finalized.

Analysis of Prognostic Markers

Analyses for leukemia cell expression of ZAP70 and of CD38 and *IGHV* mutation status were performed on peripheral blood leukemia-cell samples by the CRC Tissue Core as described.[8, 18] ZAP70 and CD38 levels were considered positive when >20% or >30%, respectively, of the circulating CLL cells expressed the marker; *IGHV* mutation status was considered unmutated when B cells expressed an *IGHV* gene with 98% homology to the most similar germline gene. Fluorescence in situ hybridization (FISH) was performed at participating CRC institutional clinical laboratories and evaluated using the hierarchical Dohner classification[11]. If subjects had multiple time points for a marker, data obtained closest to the time of enrollment in the current study were used.

^2H_2O Labeling Protocol

Measurement of CLL-cell kinetics was done at study entry. The protocol for oral administration of ^2H_2O and blood and saliva sampling was reported previously[19]. Briefly, subjects drank ^2H_2O (70% enriched) for 6 weeks: 50 ml thrice daily for a 5-day loading period followed by a 60 ml once daily maintenance phase for a total of six weeks. Target plateau body ^2H_2O enrichment was 1–1.5%. Saliva samples were obtained at weeks 2 and 4 to determine body water enrichment and ensure compliance with ^2H_2O intake. Six subjects provided weekly saliva samples (weeks 0–12) for more detailed water modeling. Blood samples were obtained at weeks 0, 3, and 6 (^2H_2O labeling phase) and at weeks 9, 12 and 16 (^2H_2O washout phase).

CLL Cell Isolation

CLL cells were isolated as described previously using the RosetteSep™ method.[19] Cell purity was determined by flow cytometry by gating on $CD19^+CD5^+$ cells. For samples that

did not achieve 95% purity, cells were fixed and isolated by FACS[19] to achieve 95% purity for CD19$^+$CD5$^+$ cells.

Measurement and Calculation of Body ^2H_2O Enrichment and ^2H-Incorporation into Purine dR of DNA

Body water ^2H_2O enrichment was determined from saliva and serum using cycloidal mass spectrometry as described.[20, 19] ^2H enrichment (atom percent excess, APE) in purine dR from DNA used for the calculation of CLL-cell kinetics was determined by GC-P-IRMS as described.[21, 19]

Cell Birth and Disappearance Rate Calculations

The fraction of cells that divided during the labelling period (f) was calculated for each time point using the precursor-product ratio based on isotopic enrichment in cellular DNA (product) and the time-averaged enrichment of ^2H_2O in body water (precursor, p) (See Supplementary Appendix).[21] Birth rate (f/day) was calculated by dividing f by the time interval up to each specific time point with a maximum value of 6 weeks corresponding to the duration of ^2H_2O ingestion. The highest birth rate of CLL cells observed across the time points for each individual was the birth rate used for subsequent analyses. Cell disappearance rate, representing the loss of labeled cells from the vascular compartment through cell death or recirculation to solid tissues, was calculated as described.[22, 23] We also assessed for "lag" or delayed release of previously divided,^2H-labelled cells into the sampled (vascular) compartment from a sequestered pool such as lymph nodes or spleen. This lag, previously observed by Messmer et al.[13] and by Van Gent et al.,[13, 15] becomes apparent when there is a delay in the initial rate of labeling and/or a continued increase in label enrichment (APE) in DNA from sampled CLL-cells long after subjects have ceased drinking ^2H_2O and ^2H_2O has washed out from body water pools (e.g. Supplemental Figure S3C). These labeled cells must have previously divided in a non-vascular compartment when ^2H_2O was present and later were released into the vascular compartment where they were sampled. Because of the potential biological significance of this cell trafficking, the presence or absence of a lag was assessed. Detailed descriptions of the calculation of ^2H_2O exposure, fractional DNA synthesis and birth and disappearance rates, as well as determination of lag and comparison with other methods for determining the birth rate can be found in the Supplementary Appendix.

Statistics

Associations between each candidate prognostic marker and outcome were evaluated using Fisher's exact test for categorical data and the Wilcoxon or Kruskal-Wallis rank-sum tests for continuous data comparing two or more groups.

Due to few events leading to sparse data on the primary endpoint, disease progression to treatment within two years of study entry, Firth's penalized logistic regression was used to obtain robust model estimates and 95% confidence intervals. This analysis included only those patients who were followed for at least two years or experienced progression within two years. Step-wise variable selection of candidate prognostic markers was used to identify factors associated with disease course.

A pre-specified exploratory TFS endpoint was assessed using a Cox proportional hazards model. Using recursive partitioning and regression trees, an optimal cut-point for birth rate was obtained, which best distinguished those needing treatment from those remaining untreated for these data.

Analyses were performed using R version 3.2.0 with packages *Hmisc, MASS, rpart*, and *survival* for analyses and *knitr* for reproducibility. P-values were two-sided. Corrections for multiple testing were applied using the Holm method.

RESULTS

Patients

CLL patients (n=119) were enrolled from six CRC institutions. As per protocol eligibility criteria, we excluded patients who had received prior treatment (N=2) or who had a date of CLL diagnosis more than 3 years prior to enrollment (N=10). Six subjects did not complete the ^2H_2O labeling protocol (1 progressed, 3 entered early intervention trials, 1 cited travel commitments, and 1 for an unknown reason) requiring withdrawal from the study. We were unable to determine kinetic rates for 4 additional subjects for analytical reasons related to cell purity and subsequent isotopic analyses. Clinical and demographic characteristics for the remaining 97 subjects are presented in Table 1.

Birth Rate and Cellular Kinetics

The median birth rate was 0.32% per day (range 0.07–1.31% per day, Figure 1). Median disappearance rate was 0.55% per day (range 0.00–3.60% per day). The kinetic profiles generally fell into one of three patterns: (1) equivalent birth and disappearance rates (Figure 2a), (2) more rapid disappearance than birth rates suggesting that the death/recirculation rate exceeded the birth rate (Figure 2b), and (3) continued increase in ^2H enrichment in cellular DNA after the 6-week labeling period indicative of a delayed release of previously divided cells from tissue compartments into the blood, "lag" (Figure 2c).

Eighteen subjects (19%) were determined to have a lag. Eleven subjects (11%) had a substantially greater disappearance rate than birth rate, and the remainder of subjects (70%) had roughly equivalent birth and disappearance rates or an indeterminate pattern.

Association of Birth Rate with Other Prognostic Measures

We compared birth rate with published markers of earlier treatment (ZAP70^{+}, CD38^{+}, and unmutated *IGHV*). Each was significantly associated with higher birth rates (Figure 3a–c). There was no significant association observed between birth rate and the few deleterious chromosome abnormalities (e.g. del(17p13.1) and del(11q23.3)) seen in this early stage cohort (Figure 3d; Table 2).

Progression

During a median follow-up of 4.0 (0.0–6.9) years, 33 patients (34%) required treatment (Table 1) with treatment occurring within 2 years for 14 of the 33 patients. Six patients were followed for less than two years (one died of pneumonia at day 148 and 5 were lost to

follow-up before two years) and were excluded from the logistic regression but were retained for the analyses of TFS.

Association of Individual Prognostic Markers with Disease Progression

Univariable TFS Kaplan-Meier curves illustrate a significant association with TFS for all prognostic markers studied (Figure 4). A higher birth rate was a significant predictor of TFS using both the previously established cut point of 0.35% cell/d[13] (Figure 4a) and the optimal predictive cut-point for birth rate determined in the current study (0.49% cells/d, Figure 4b). Forty-three subjects (44%) had birth rates above the 0.35% cells per day threshold. Distribution of the prognostic markers and birth rates among patients who did and did not progress to treatment are shown in Supplemental Table 1. Cellular disappearance/death rate did not associate with progression and thus was omitted from further modeling.

Logistic Model

For statistical modeling we used a cut point of >0.35% cells/d, which was previously associated with more aggressive disease[13]. Also, while not yet validated, we modeled using the optimal predictive cut point determined in this study, which was 0.49% cells/d. Firth's penalized likelihood was used for the logistic regression. Predictor variables at hand for the model included sex, age at study entry, Rai stage, birth rate, presence of a delayed appearance of labeled cells in blood (lag), CD38 or ZAP70 expression, *IGHV* mutation status and chromosomal abnormalities by FISH. In the final model, only CLL birth rate and *IGHV* mutation status contributed significantly. Odds ratios were 7.59 (95% CI: 1.24–31.15) for birth rate >0.35% cells/d and 67.07 (95% CI: 7.98–9100) for unmutated *IGHV*. Using the optimal cut point of birth rate defined in this study (>0.49% cells per day) the odds ratios were 15.53 (95% CI: 2.92–117.15) for birth rate and 82.12 (95% CI: 8.62–1100) for umuntated IGHV (Supplemental Table 2). The area under the receiver operation characteristic curve of the model showed an extremely good prediction accuracy of 0.94 with the 0.35% per day cut point and of 0.96 with the 0.49% per day cut point.

Time to Event Model

A stepwise Cox proportional hazards model using all available data was used to investigate the relationships with TFS. Again, only cell birth rate and *IGHV* mutation status contributed significantly. Hazard ratios were 3.51 (95% CI: 1.61–7.67; p=0.002) for CLL birth rate >0.35% cells/d and 4.93 (95% CI: 2.41–10.12; p <0.001) for unmutated *IGHV*. Using the optimal cut point of birth rate of >0.49% cells per day, the hazard ratios were 6.43 (95% CI: 3.10–13.34) for fast birth rate and 4.87 (95% CI: 2.37–10.00) for unmuated *IGHV* (Supplemental Table 3). Kaplan-Meier analyses using 0.35% per day depict four separable curves and show that higher birth rate is associated with shorter TFS in both the unmutated and the mutated *IGHV* groups (Figure 5).

DISCUSSION

In this blinded, prospective study of recently diagnosed patients with early stage CLL, we found that a higher CLL-cell birth rate significantly associated with a shorter TFS, thereby providing a new prognostic marker in early stage patients that is linked directly to the

biology of the disease. Though the cut-point of 0.35% was from a cross-sectional study with a heterogeneous patient population[13], it discriminated well between shorter and longer TFS. The optimal predictive cut-point of 0.49% cells/d determined in the present data set may be a better discriminator in patients with early stage disease, although this will need to be validated in future studies.

While failed cell death no doubt remains an important contributor to CLL,[16] our data confirm findings from smaller studies[13–15] demonstrating that a proliferative compartment exists in CLL clones and that the cellular birth rates within this compartment differ among subjects (~20-fold here). Birth rates for the early stage, untreated patients studied here ranged from 0.07% to 1.31% per day, with the latter birth rates being greater than those observed for normal B cells[14, 15].

We calculated birth rate using a simplified clinical protocol and assumed a single homogeneous pool of cells with a steady-state pool size. This is an oversimplification as cellular kinetics for both normal polyclonal B lymphocytes and for CLL cells have multiple potential and established biologic complexities.[22] As noted in 1968 by Zimmer et al[24], and confirmed more recently[25], CLL cell populations are heterogeneous with distinct immunologic and anatomic pools having different kinetic rates. Although we sampled cells from the vascular compartment, lymph nodes, spleen and bone marrow contain a large number of cells that can traffic into and out of the vascular compartment. Indeed, it has been suggested that these anatomic sites vary in the numbers of proliferating cells within them[26]. Moreover, the potential delay in entry of newly proliferated cells into the vascular compartment and the transit time or lag from birth to mobilization from tissue (Figure 2c) introduces another variable, which was observed in almost 20% of the subjects in this study. Despite these biological complexities, the relatively simple method presented here for calculation of birth rate provided results that aligned closely with more complex mathematical modeling and has the advantage of more accurately calculating birth rates in patients with a lag. Most importantly, the use of a previously established method for the calculation of birth rate allowed us to use the previously published cut point for birth rate of 0.35% cells per day,[13] for prospective binary classification of subjects as slow or fast proliferators in the current clinical outcomes trial.

The strong association of short TFS with elevated proliferation rate of the total pool of CLL cells and the lack of association with lag or disappearance rate suggest that total cell proliferation per se is a significant factor in CLL disease progression. A relatively high rate of DNA replication may enable more rapid clonal evolution, allowing for leukemia-cell acquisition of new genetic aberrations conducive to more aggressive disease[27]. Future studies designed to explore the complexities of CLL kinetics and associated new genomic abnormalities may provide a more detailed understanding of the biology of CLL.

Each of the prognostic markers studied here was a predictor of TFS in univariable modeling and, with the exception of FISH, was significantly associated with leukemia cell birth rate. The strong association between higher birth rate and unmutated *IGHV* gene status in subjects with newly diagnosed early-stage disease is consistent with results from a cross-

sectional study of 9 subjects with Rai stage 0–IV disease where an inverse correlation was observed between birth rate and *IGHV* mutation levels[15].

In multivariable modeling, however, we found that only birth rate >0.35% cells/d and unmutated *IGVH* mutation status were significant contributors to disease progression with hazard ratios for shorter TFS of 3.5 and 4.9, respectively. The lack of other prognostic markers as independent contributors to the model is consistent with the hypothesis that prognostic markers based on the immunobiology of CLL cells (intracellular ZAP70 and surface membrane CD38 expression) are real time indicators of clonal dynamics and disease aggressiveness[28]. These markers therefore may overlap, at least partially, with the leukemia cell proliferation rates measured in this study. *IGHV* mutation status, on the other hand, is a static marker at the clonal level that represents the maturation pathway the cell travelled in its transition to leukemia.[29] Recent studies showing greater long-term disease free survival with treatment (fludarabine/cyclophosphamide/rituximab) in *IGHV*-mutated as opposed to unmutated disease[30–32] suggest that these early disease markers also may have implications for treatment response.

Strengths of this study include its prospective and blinded nature that focused on patients within only a few years of diagnosis that as yet did not have clinical findings suggestive of disease progression. It is this clinical group for whom determination of prognosis is most relevant. In addition, TFS was defined from the time of the measurement of the prognostic markers, including birth rate, rather than from time of diagnosis of the disease. A potential limitation is that the patients in this study were enrolled at major referral centers and had a median age at diagnosis of 57 years, which is substantially younger than that of patients evaluated in the community setting (~70 years). Nonetheless, the decision for early intervention can be a more pressing concern for younger patients, who might benefit most from the knowledge that they are at risk for a more aggressive disease course.

The protocol for ^2H_2O labeling and sample collection used here represents a considerable improvement in terms of clinical feasibility compared with more complex previous approaches.[13, 19] Strategies such as the use of stable isotope labeled glucose may further reduce labeling time and blood sampling, thereby improving patient compliance and resulting in a more clinically usable assay.[33]

In conclusion, increased birth rate of CLL cells early in the disease course is a strong predictor of the need for earlier initial treatment reinforcing the concept that enhanced cell proliferation is an important driver in the biology of disease progression. Future studies monitoring proliferation rates over time, their response to treatment, and relation to survival will further expand our understanding of the role of CLL-cell kinetics in this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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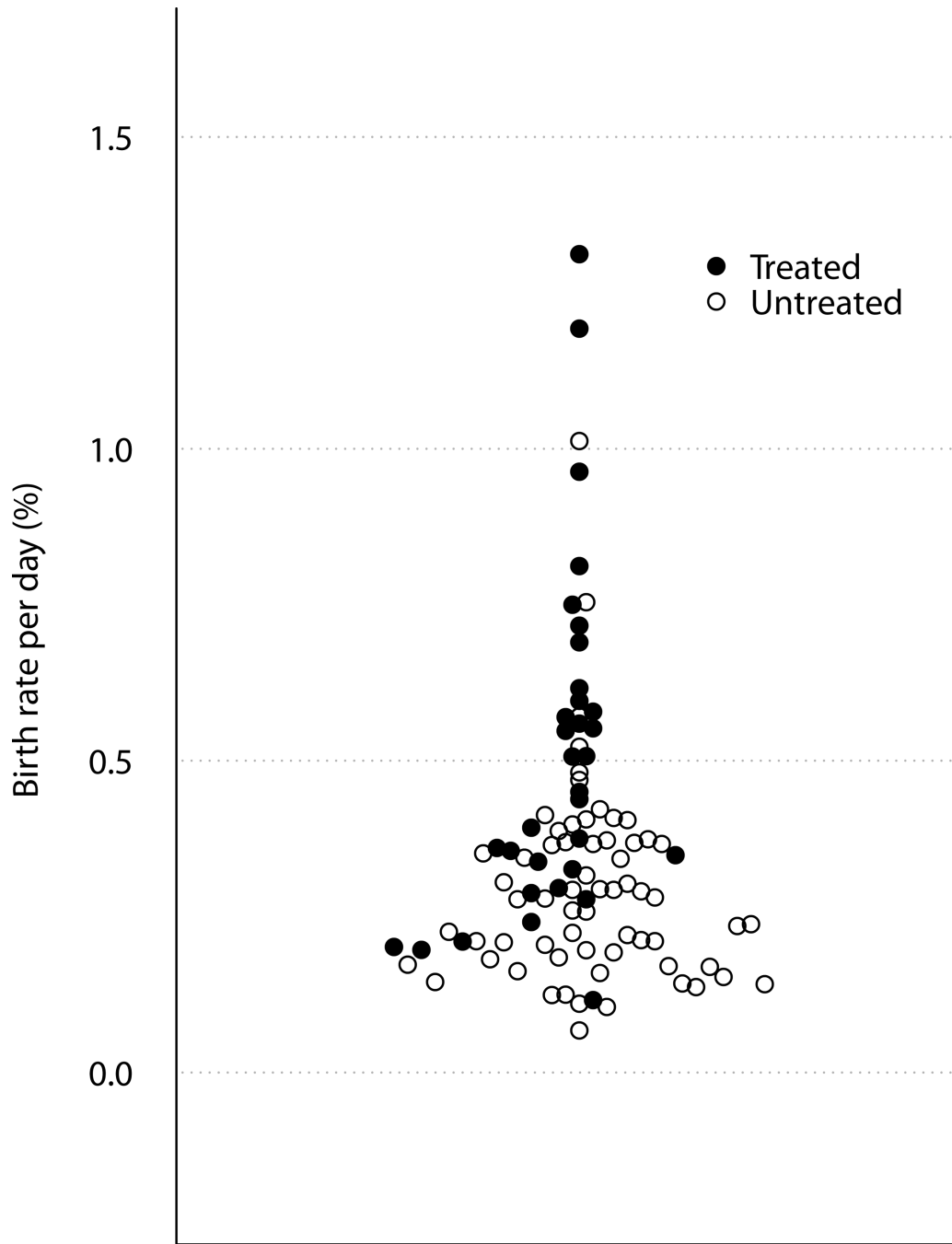
Figure 1. CLL Cell Birth Rate

Birth rate (% new cells per day) in treated (closed circles) and untreated (open circles) for early stage CLL patients (n=97).
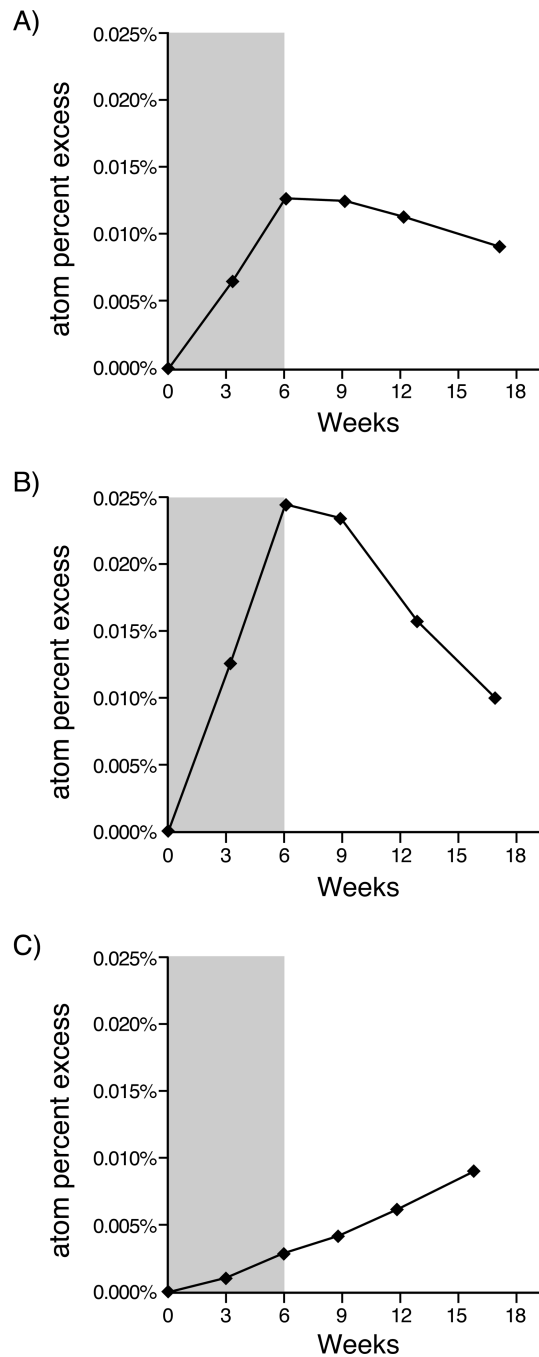
Figure 2. CLL Cell Kinetics

Atom percent excess representing ^{2}H enrichment in DNA of CLL cells over time in three individual patients; six week period of ^{2}H$_2$O labeling is indicated by the shaded area. A) pattern consistent with similar birth and disappearance rates compatible with a simple single pool system B) rapid birth rate and more rapid disappearance rate C) delayed release (lag) of previously labeled cells into the vascular compartment many weeks after discontinuation of ^{2}H$_2$O.
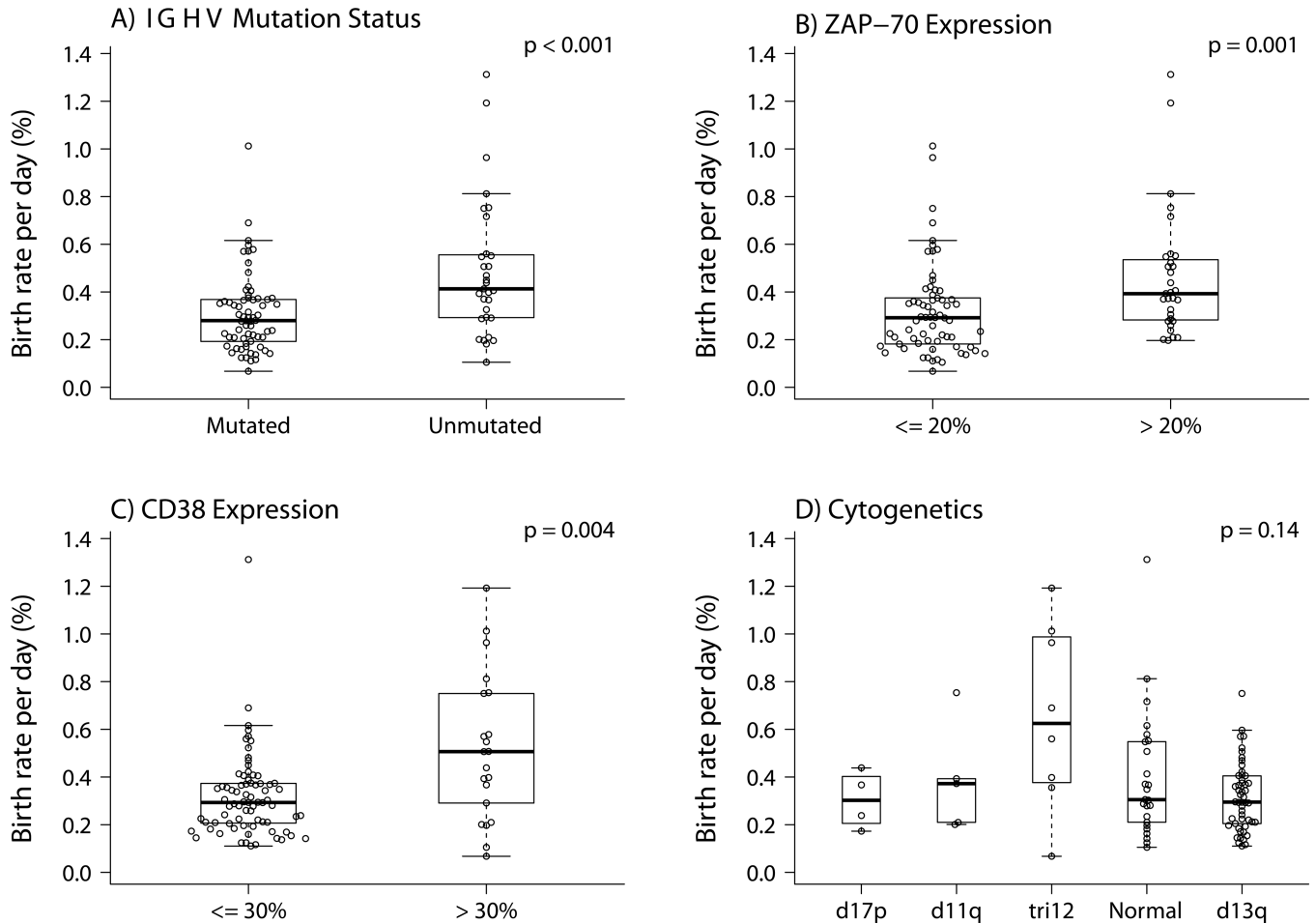
Figure 3. Birth Rate in Relation to Other Prognostic Factors
A) Shows birth rate is significantly higher for patients with unmutated versus mutated *IGHV*s (n=97). B) Shows birth rate is significantly higher for patients with high levels of ZAP70 expression (n=97). C) Shows birth rate is significantly higher for patients with high levels of CD38 expression (n=96). D) Shows the relationship between birth rate and cytogenetic findings by FISH (n=94). With relatively small numbers for the most deleterious mutations (e.g. 17p and 11q deletions), there was no significant association with birth rate.
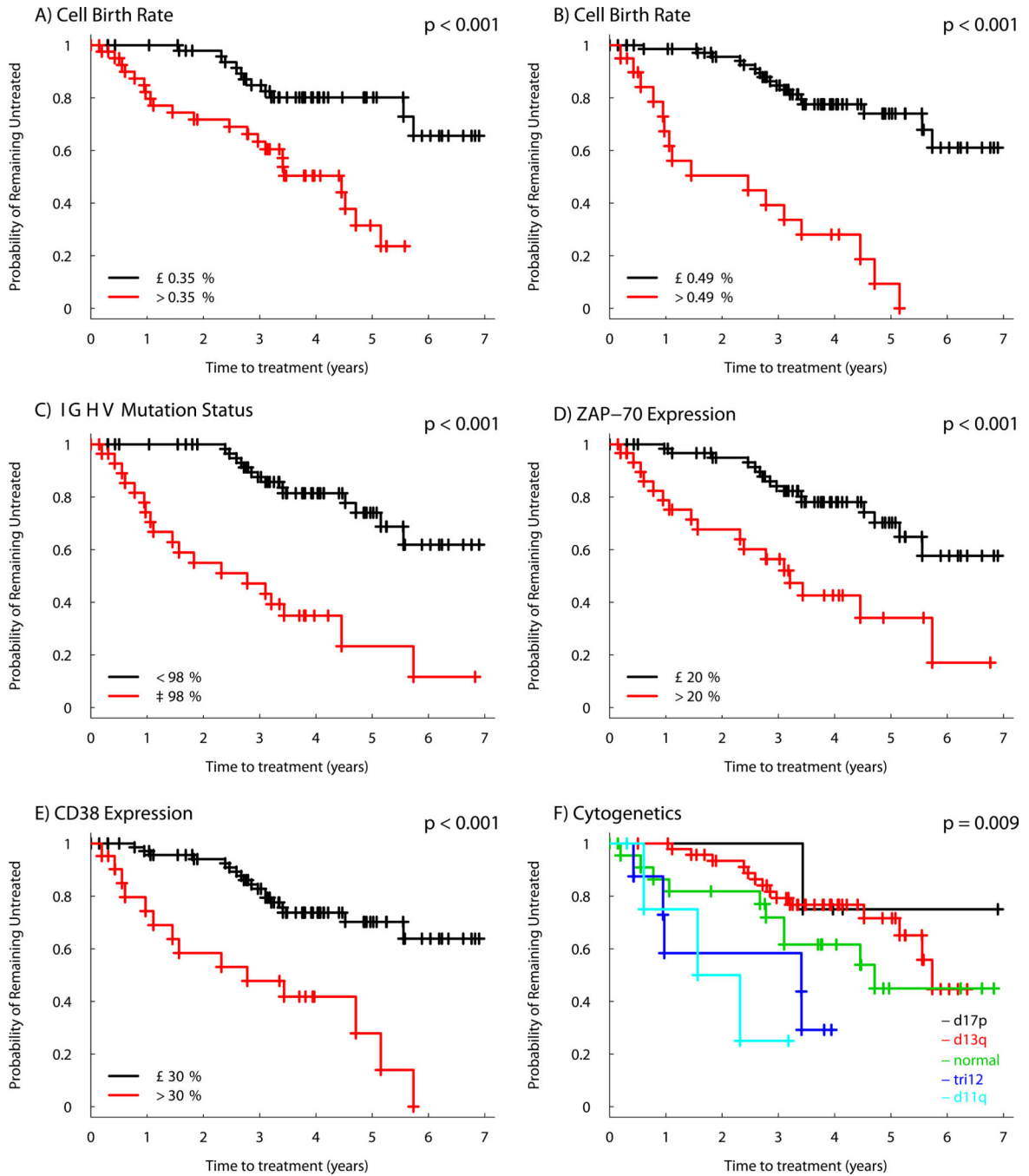
Figure 4. Relationship between Prognostic Markers and Time from Measurement to Initial Therapy

Kaplan-Meier curves depict the proportion of untreated patients with CLL according to the time from measurement of the prognostic marker for A) birth rate > 0.35% per day, B) birth rate > 0.49% per day, C) *IGHV* 98% homology, D) ZAP70 expression > 20%, E) CD38 expression > 30%, F) Dohner hierarchical categorization of FISH cytogenetics.
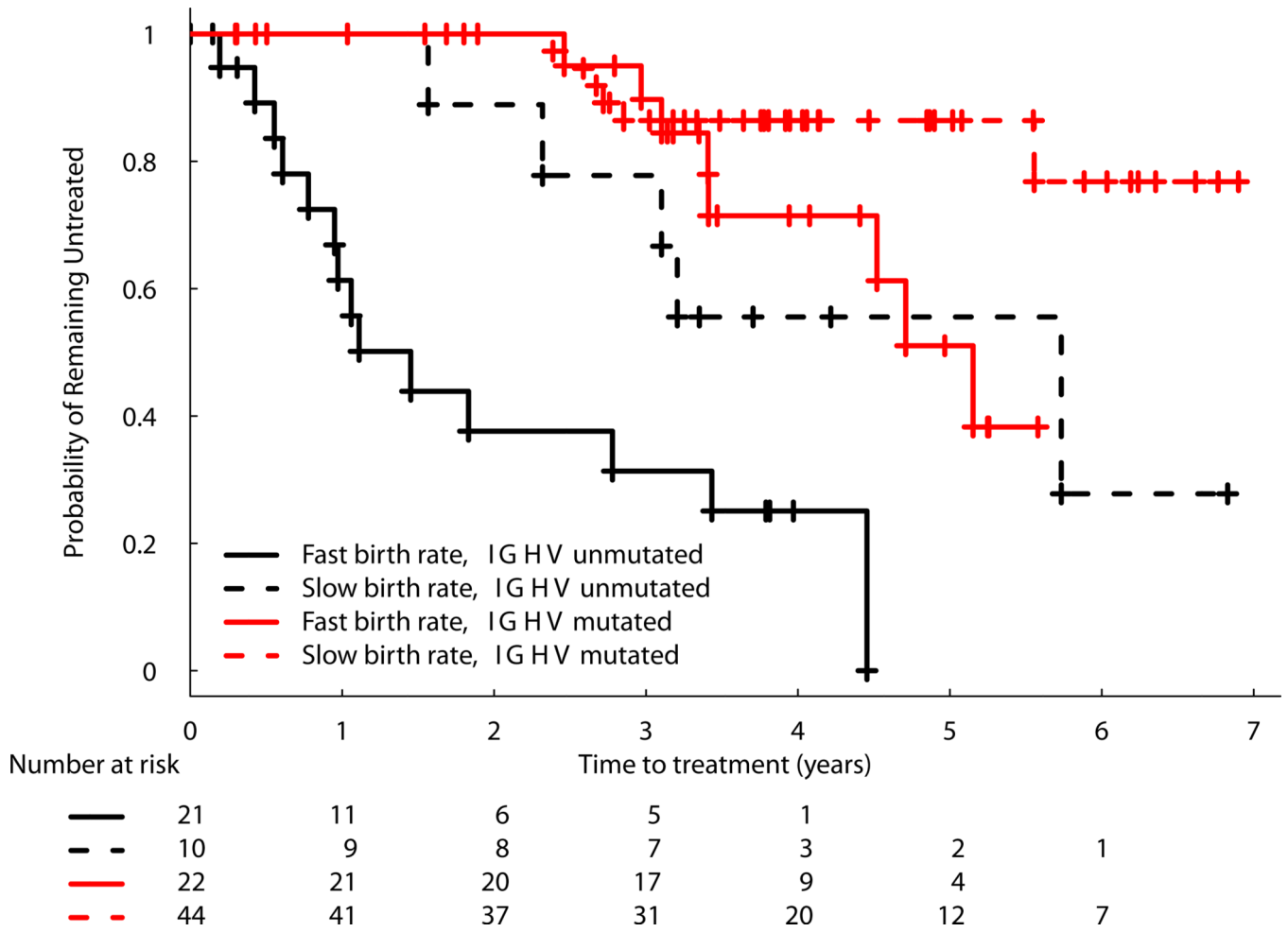
Figure 5. Kaplan-Meier Curve of TFS Model Stratified by *IGHV* Mutation Status and CLL-Cell Birth Rate

The curves shown represent the proportion of untreated patients with CLL since time of entry into the study. Patients are stratified into 4 categories: fast birth rate (>0.35% per day), unmutated *IGHV* (98% homology); slow birth rate, unmutated *IGHV*; fast birth rate, mutated *IGHV*; and slow birth rate unmuated *IGHV*.

Table 1

Study Participant Demographics and Treatment Outcome

		Treated CLL	
	Total n = 97	**No n = 64**	**Yes n = 33**
Sex – no.(%)			
Female	39 (40%)	26 (41%)	13 (39%)
Male	58 (60%)	38 (59%)	20 (61%)
Age –yr			
Median (range)	57 (40 – 85)	58 (40 – 85)	56 (41 –78)
Race – no. (%)			
White	92 (95%)	60 (94%)	32 (97%)
Asian	1 (1%)	1 (2%)	0 (0%)
Multi-Racial	1 (1%)	1 (2%)	0 (0%)
Unknown	3 (3%)	2 (3%)	1 (3%)
Rai stage at enrollment – no. (%)			
0: Lymphocytosis only	36 (37%)	32 (50%)	4 (12%)
I: Lymphocytosis with lymphadenopathy	44 (45%)	24 (38%)	20 (61%)
II: Lymphocytosis with hepatomegaly or splenomegaly	17 (18%)	8 (12%)	9 (27%)
Enrollment site – no. (%)			
Dana-Farber Cancer Institute	5 (5%)	3 (5%)	2 (6%)
North Shore - Long Island Jewish Health System	19 (20%)	11 (17%)	8 (24%)
Mayo Clinic	18 (19%)	12 (19%)	6 (18%)
MD Anderson Cancer Center	14 (14%)	9 (14%)	5 (15%)
Ohio State University	10 (10%)	9 (14%)	1 (3%)
University of California San Diego	31 (32%)	20 (31%)	11 (33%)
CLL diagnosis to study entry -months			
Median (range)	12 (1 – 36)	11 (1 – 36)	12 (1 – 36)

Table 2

Chromosomal aberration by FISH for 97 eligible patients.

Treatment for CLL	Abnormality						
	d17p	d11q	tri12	Normal	d13q	ND[†]	Total
No	3	2	3	16	37	3	64
Yes	1	3	5	10	14	0	33
Total	4	5	8	26	51	3	97

[†]Three patients who did not have FISH data are noted as ND (not determined)