Incidence and risk factors of bleeding-related adverse events in patients with chronic lymphocytic leukemia treated with ibrutinib

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ABSTRACT

Ibrutinib is associated with bleeding-related adverse events of grade ≤2 in severity, and infrequently with grade ≥3 events. To investigate the mechanisms of bleeding and identify patients at risk, we prospectively assessed platelet function and coagulation factors in our investigator-initiated trial of single-agent ibrutinib for chronic lymphocytic leukemia. At a median follow-up of 24 months we recorded grade ≤2 bleeding-related adverse events in 55% of 85 patients. No grade ≥3 events occurred. Median time to event was 49 days. The cumulative incidence of an event plateaued by 6 months, suggesting that the risk of bleeding decreases with continued therapy. At baseline, von Willebrand factor and factor VIII levels were often high and normalized on treatment. Platelet function measured via the platelet function analyzer (PFA-100™) was impaired in 22 patients at baseline and in an additional 19 patients on ibrutinib (often transiently). Collagen and adenosine diphosphate induced platelet aggregation was tested using whole blood aggregometry. Compared to normal controls, response to both agonists was decreased in all patients with chronic lymphocytic leukemia, whether on ibrutinib or not. Compared to untreated chronic lymphocytic leukemia patients, response to collagen showed a mild further decrement on ibrutinib, while response to adenosine diphosphate improved. All parameters associated with a significantly increased risk of bleeding-related events were present at baseline, including prolonged epinephrine closure time (HR 2.74, P=0.012), lower levels of von Willebrand factor activity (HR 2.73, P=0.009) and factor VIII (HR 3.73, P=0.0004). In conclusion, both disease and treatment-related factors influence the risk of bleeding. Patients at greater risk for bleeding of grade ≤2 can be identified by clinical laboratory tests and counseled to avoid aspirin, non-steroidal anti-inflammatory drugs and fish oils. *ClinicalTrials.gov identifier NCT01500733*

Introduction

Ibrutinib (Imbruvica) is an inhibitor of Bruton's tyrosine kinase (BTK). BTK is a cytoplasmic tyrosine kinase of the TEC family that is essential for B-cell receptor signaling.[1-4] Ibrutinib has significant activity in chronic lymphocytic leukemia (CLL),[5] mantle cell lymphoma (MCL)[6] and Waldenström's Macroglobulinemia (WM),[7] and its use is being explored in other B-cell malignancies.[2,8,9] Overall response rates of >70%, and an estimated 26-month progression-free survival rate of 75% for previously treated patients with CLL have been reported.[10] Ibrutinib received FDA approval as a second-line therapy for MCL in November 2013 and for CLL in February 2014. In July 2014 approval was expanded to all CLL patients with the chromosome 17p13.1 deletion. Although ibrutinib is well-tolerated, early clinical studies reported grade ≥3 bleeding events including subdural hematomas, hematuria, and gastrointestinal bleeding in 5-6% of patients,[10] often in those with other bleeding risk factors such as warfarin use. Subsequent trials have therefore excluded patients on warfarin, and it is recommended that ibrutinib be held prior to invasive procedures. Studies also documented frequent grade 1 ecchymosis, with one recent multi-center trial reporting petechiae and ecchymoses in 44% of patients compared to 12% in the control arm of ofatumumab, with no difference in grade 3 bleeding.[11]

Given that most patients with CLL are elderly, many who are candidates for ibrutinib therapy will have comorbidities requiring anticoagulation and/or antiplatelet therapy. For example, one study reported atrial fibrillation in 5% of patients on ibrutinib.[11] Therefore, understanding the mechanism by which ibrutinib may contribute to bleeding and identifying possible predictors for such adverse events is important for clinical management.

While it is established that BTK plays a role in glycoprotein signaling in platelets through GPIb and GPVI,[12-14] the clinical significance of BTK inhibition in platelets is less clear. Notably, patients with X-linked agammaglobulinemia (XLA), an immunodeficiency syndrome caused by inactivating mutations in BTK, do not exhibit increased bleeding.[13] The apparent discrepancy in bleeding propensity between patients with hematologic malignancies treated with ibrutinib and patients with XLA who lack BTK is intriguing and suggests that further investigation is required to understand how ibrutinib

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may contribute to bleeding. Therefore, we prospectively recorded bleeding-related adverse events in patients with CLL treated with single-agent ibrutinib, monitored platelet counts, prothrombin time (PT), activated partial thromboplastin time (aPTT), factor VIII (FVIII) and von Willebrand factor (vWF) antigen levels and function and assessed platelet function using standard clinical testing.

Methods

Patients and treatment

85 patients with CLL (53 previously untreated patients and 32 with relapsed or refractory disease) were enrolled from December 2011 through May 2013 in our investigator-initiated phase 2 study of ibrutinib (NCT01500733). The study was approved by the NHLBI institutional review board (12-H-0035). A platelet count of ≥ 30,000/μL at enrollment was required. Exclusion criteria included transformed disease, autoimmune hemolytic anemia or thrombocytopenia requiring steroid therapy, significant impairment of hepatic or renal function, concomitant corticosteroid treatment equivalent to prednisone >20mg/day, and anticoagulation with warfarin. Patients received 420 mg of ibrutinib once daily on 28 day cycles consecutively, until disease progression or intolerable side effects occurred. Safety monitoring was done every other week for the first month, monthly until month 6, and every 3 months thereafter. Data cut-off was August 2014. The primary endpoint, overall response at 24 weeks, is reported elsewhere.[15]

Samples from treatment-naïve CLL patients (*ClinicalTrials.gov Identifier: NCT00019370*) were used as controls in whole blood aggregometry studies. Patients with XLA were enrolled on protocol 93-I-0119 at the National Institute of Allergy and Infectious Diseases. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Laboratory Methods

We prospectively measured platelet count, platelet function assays (PFA-100™), vWF activity and antigen levels, factor VIII levels, PT, and aPTT at baseline and on days 2 and 28. Laboratory studies were performed in the NIH Clinical Center Department of Laboratory Medicine. Closure times were measured on the PFA-100™ instrument (Siemens Healthcare, Malvern, PA) using epinephrine (EPI) and ADP impregnated collagen membrane cartridges. Only samples with a platelet count >100K/μL and hematocrit level >35% were analyzed. In a subset of the ibrutinib-treated patients, treatment-naïve CLL control patients, and XLA patients, we measured platelet aggregation with collagen and ADP agonists in 0.105 M sodium citrate-anticoagulated whole blood samples via impedance aggregometry and platelet dense granule ATP release by chemiluminescence on a Chrono-log 700 lumi-aggregometry instrument (Havertown, PA). Representative tracings were processed using the center trace function in CorelDRAW X6 (Corel Corporation, Ottawa, ON, Canada).

Outcomes

Bleeding-related adverse events were defined as any patient-reported bleeding or bruising, or any documentation of bleeding or bruising upon physical examination. Included in the analysis were events deemed treatment-related, and graded according to common terminology criteria for adverse events (CTCAE) version 4.03. Minor bruising resulting from lymph node biopsy was not counted as an event unless deemed excessive or persisting for > 2 weeks post procedure. Ibrutinib therapy was not held prior to lymph node or bone marrow biopsies.

Statistical analysis

Summary statistics such as mean, standard error of the mean (SEM), median, and interquartile range (IQR) were presented and compared between subgroups of patients with or without bleeding events. Continuous variables were compared using a t-test or Wilcoxon rank-sum test as appropriate, and categorical variables were compared using the Fisher's exact test. We analyzed the relationship between clinical characteristics, laboratory values, and bleeding events utilizing both univariate and multivariate Cox proportional hazards regression models using SAS software version 9.3 (SAS Institute Inc., Cary, NC). Platelet function variables were evaluated both as continuous variables and as dichotomized variables using the best cut-off values for discriminating bleeding events. A two-sided *P* value of less than 0.05 was considered statistically significant.

Results

Incidence and presentation of bleeding events on ibrutinib

47 out of 85 patients (55%) had 75 documented bleeding-related adverse events at a median follow-up of 24 months (IQR 12.9-26.4). All bleeding events were mucocutaneous in nature; they included bruising/petechiae/ecchymoses (62 events), epistaxis (8 events), subconjunctival bleeding (3 events) and oral/gingival bleeding (2 events). 71 out of 75 (95%) events were grade 1 in severity. Four grade 2 events (5%) were observed, including one instance of ecchymosis, one subconjunctival bleed and two instances of epistaxis. No grade ≥ 3 events occurred. The *Online Supplementary Table S1* summarizes all bleeding events. The median time to onset of the event was 49 days (IQR 13-108). 22 out of 47 (47%) of the patients who experienced bleeding did so within the first month (Figure 1A). 34 out of 75 (45%) of the events were still ongoing at data cutoff. For the 55% of events that resolved during follow-up, the median time to resolution was 28 days (IQR 14-51.5). Of the 47 patients with bleeding, 9 (19%) were on aspirin, 6 (13%) used other NSAIDs, 6 (13%) were taking fish oil, 1 transiently used dabigatran, and another patient transiently used a factor Xa inhibitor (apixaban). The relative risk of an event for patients taking any of these medications was 1.45 (95% confidence interval (CI) 0.98 to 3.59, *P*=0.081 by Fisher's exact test). There were no statistically significant differences in baseline characteristics (including age, treatment indication, or disease stage) between patients who experienced bleeding and those who did not (Table 1).

Before Pharmacylics advised investigators to hold ibrutinib prior to invasive procedures, we performed over 30 lymph node biopsies on day 2 or day 4 as part of our original protocol design without any grade ≥2 bleeding.

Platelet counts

At baseline, 44 out of 85 patients (52%) had grade 1 thrombocytopenia (platelet count < lower limit of normal but ≥75k/μL), 14 (16%) had grade 2 (<75k/μL) and 9 (11%) had grade 3 (<50k/μL) thrombocytopenia. After initiation of ibrutinib therapy, the majority of patients showed a small decrease in platelet counts on day 2 (median decrease 5k/μL, IQR 5-14) followed by a rapid increase in platelet count (Figure 1B). 8 patients with platelet counts <50k/μL were enrolled on the study, and in two of these the platelet count transiently decreased to <25k/μL. After

4 weeks (one cycle) of treatment in 7 out of 8 (88%) patients enrolled with platelet counts <50k/μL, the count increased to >50k/μL (median 102k/μL, IQR 61-164). 25 out of 37 (68%) of the patients who started treatment with a baseline platelet count <100k/μL exceeded that threshold by 6 months (median 104k/μL, IQR 82-152). In summary, platelet counts typically improved on ibrutinib, and there was no significant difference in platelet counts between patients with or without bleeding at baseline or on day 28 (Figure 1C).

Changes in vWF and FVIII

Since a decrease in vWF (acquired type 1 von Willebrand's disease) has been described in patients with lymphoproliferative disorders with a high lymphocyte count, we investigated whether the treatment-induced lymphocytosis on ibrutinib elicits a similar effect.[10,16] We prospectively measured vWF antigen, vWF activity, and FVIII levels in all patients prior to initiation of ibrutinib therapy and on day 28 (Figure 2A-C). Interestingly, at baseline many patients had levels above normal. On treatment the majority of patients had a notable decrease in all three parameters, reaching normal levels on day 28. vWF multimer analysis in 7 patients (*data not shown*) revealed normal size distribution of multimers, consistent with a non-selective decrease in vWF of all sizes. Of note, there was no correlation between the decrease in vWF antigen and increase in lymphocytosis. Additionally, there was no PT or aPTT prolongation in any of our patients.

Platelet function testing

We prospectively performed PFA-100™ testing using both epinephrine (EPI) and adenosine diphosphate (ADP) agonist cartridges in 66 out of the 85 (78%) patients enrolled. The assay was not performed in the remainder due to either thrombocytopenia (<100,000/μL), clumping induced by leukocytosis, or hematocrit level <35%. 41 out of the 66 (62%) patients tested had prolongation in EPI closure time at one or more timepoints. 22 patients showed prolonged EPI closure times at baseline, and 11 of these used medications known to affect platelet aggregation, including antiplatelet agents or anticoagulants (n=8), and fish oil or garlic supplementation (n=3). 19 patients (28%) had normal EPI closure times at baseline that became prolonged on ibrutinib, and were considered to be possibly treatment-related. However, in 12 patients this was transient, alternating between normal and prolonged. For the entire cohort, ADP closure times were either low or normal (*data not shown*). Prolonged EPI closure time at baseline was associated with an increased risk of bleeding (hazard ratio per minute increase 1.34, 95% CI, 1.02-1.75, P=0.031). In contrast, prolongation in EPI closure time after initiation of ibrutinib was not associated with increased risk of bleeding (RR = 0.91, 95% CI 0.497 to 1.669, P=0.76).

Predicting bleeding risk on ibrutinib

To investigate the relationship between laboratory measurements and bleeding risk we explored all variables

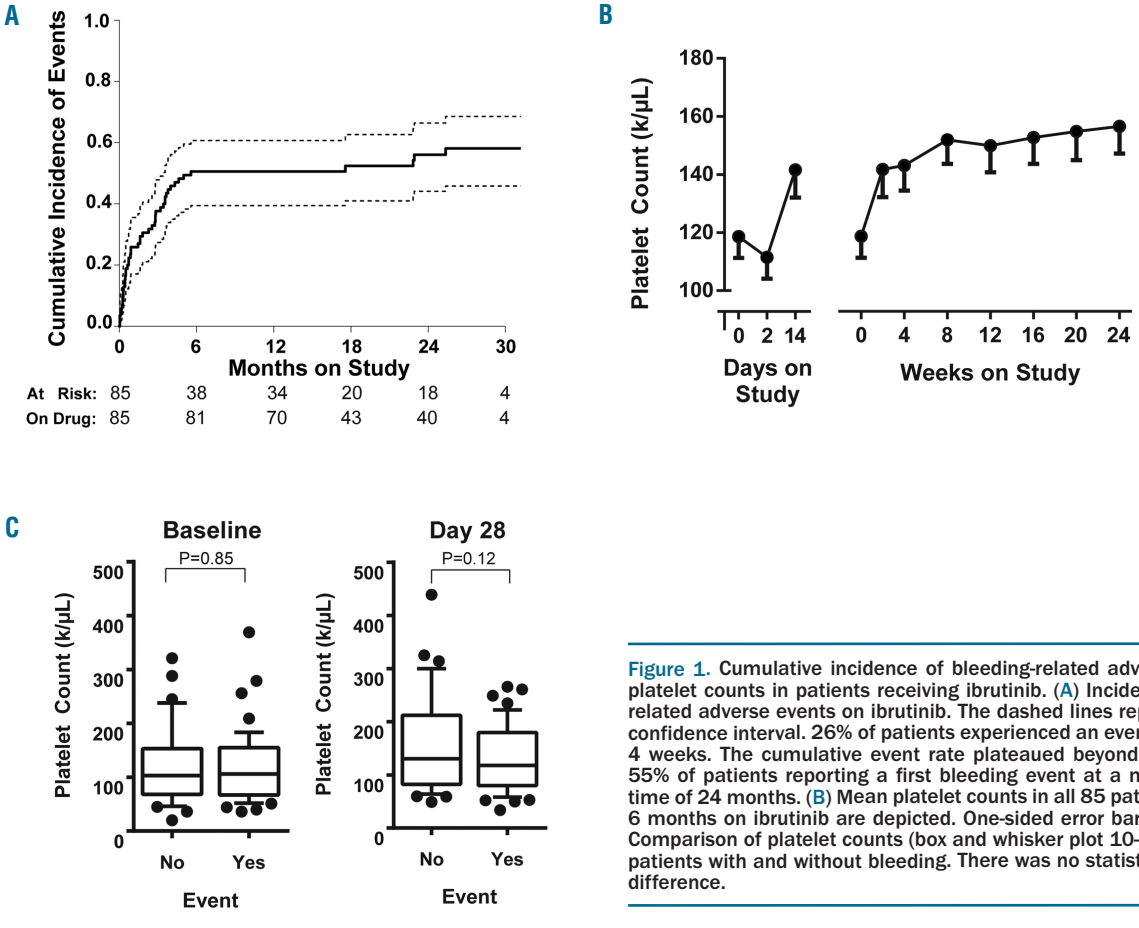

Figure 1. Cumulative incidence of bleeding-related adverse events and platelet counts in patients receiving ibrutinib. (A) Incidence of bleeding-related adverse events on ibrutinib. The dashed lines represent the 95% confidence interval. 26% of patients experienced an event within the first 4 weeks. The cumulative event rate plateaued beyond 24 weeks, with 55% of patients reporting a first bleeding event at a median follow-up time of 24 months. (B) Mean platelet counts in all 85 patients for the first 6 months on ibrutinib are depicted. One-sided error bar shows SEM. (C) Comparison of platelet counts (box and whisker plot 10-90 percentile) in patients with and without bleeding. There was no statistically significant difference.

Table 1. Characteristics of study participants.

Characteristic	All patients (n=85)	Patients with events (n=47)	Patients without events (n=38)	*P*
Median age (range) - yr	65.8 (33-85)	67 (39-81)	65 (33-85)	0.29
Male - no. (%)	49 (58)	26 (55)	23 (61)	0.66
Median hemoglobin (range) g/dL	10.95 (5.8-13.9)	11.2 (8.5-13.9)	10.45 (5.8-13.9)	0.24
Median platelet count (range) per mm^3	103 (30-369)	103 (30-321)	106 (36-369)	0.99
Median ALC (range) per mm^3	87 (0.31-402)	83 (0.48-281)	89 (0.31-402)	0.45
Rai Stage				>0.99
1 - 2	27 (32)	15 (32)	12 (32)	
3 - 4	58 (68)	32 (68)	26 (68)	
Mutation Status - unmutated	51 (65)	27 (61)	24 (69)	0.51
Cytogenetic abnormalities				
17p deletion	51 (60)	28 (60)	23 (60)	>0.99
11q deletion	20 (23)	8 (17)	12 (32)	0.13
Trisomy 12	13 (15)	6 (13)	7 (18)	0.55
13q deletion	49 (58)	28 (60)	21 (55)	0.82
Previously treated	32 (38)	18 (38)	14 (36)	>0.99
Indication to start treatment				
Bulky lymphadenopathy	8 (9)	3 (6)	5 (13)	0.46
Constitutional symptoms	32 (38)	17 (36)	15 (39)	0.82
Cytopenias	57 (67)	33 (70)	24 (63)	0.64
Lymphocyte doubling time	5 (6)	3 (6)	2 (5)	>0.99

Continuous variables were compared by the Wilcoxon rank-sum test and categorical variables were compared by the Fisher's exact test.

for significant associations with bleeding using univariate Cox regression analysis. Continuous variables with $P<0.15$ were dichotomized to examine possible nonlinear relationships (*Online Supplementary Table S2*). Prolongation of EPI closure time at baseline was associated with increased bleeding risk. After dichotomizing this variable, an elevation of ≥240 seconds (normal range 86-154 seconds), present in 10 patients, was associated with a hazard ratio (HR) of 2.70 (95% CI, 1.26-5.79, $P=0.008$, Figure 3A). Univariate analysis of Factor VIII and vWF activity levels in 77 patients revealed that lower levels of FVIII and of vWF activity at baseline demonstrated a trend towards increased bleeding. Dichotomizing Factor VIII and vWF activity levels using cut-offs of ≤174 IU/dL (reference range 41-184 IU/dL) and ≤100 IU/dL (reference range 52-156 IU/dL) showed an association with increased risk of bleeding (for FVIII, HR 2.89, $P=0.0007$, for vWF activity HR 2.25, $P=0.017$, *Online Supplementary Table S2*, Figure 3B-C).

Next we performed multivariate Cox-regression analysis after controlling for potential confounders, including age and prior treatment status (Table 2). Predictors of bleeding found to be statistically significant in the multivariate analysis were EPI prolongation (HR 2.74, $P=0.012$), relatively lower FVIII levels (HR 3.73, $P=0.0004$) and vWF activity at baseline (HR 2.73, $P=0.009$), dichotomized as described above. We also analyzed an iteration of the model that combined these three parameters in the subset of patients for whom complete data was available (n=60), demonstrating either a trend or significant association with increased risk of bleeding or bruising (Table 2, Model 4).

Collagen-induced platelet aggregation and dense granule release

BTK is expressed in platelets and activated in response to collagen. Other studies reported inhibition of collagen-mediated platelet aggregation by ibrutinib in platelet rich plasma and concluded that this effect may increase bleeding risk on ibrutinib.[17-19] Since CLL cells themselves have been reported to modulate platelet activation[20] we studied collagen-induced platelet aggregation using whole blood aggregometry in patients on ibrutinib, untreated CLL patients, healthy controls, and patients with XLA. Representative tracings from collagen-mediated aggregometry are shown in *Online Supplementary Figure S1*.

Compared to normal controls, collagen-mediated aggregation was significantly decreased in all patients with CLL whether on treatment with ibrutinib or not (Figure 4A); untreated CLL patients had a 43% reduction in response to low dose (1 μg/ml) collagen ($P<0.0001$) and an 18% reduction in response to high dose (5 μg/ml) collagen ($P=0.048$). Compared to untreated CLL patients, patients on ibrutinib had a slight further decrement in response to collagen of 21% at low dose ($P=0.101$) and 20% at high dose ($P=0.041$). Compared to XLA patients, CLL patients on ibrutinib had a slight reduction in response to low dose collagen and essentially the same level of aggregation at high dose collagen (Figure 4A). Collagen-induced release of ATP from dense granules was significantly impaired in CLL patients and ibrutinib-treated CLL patients compared to controls (Figure 4B). In contrast, the release of ATP from dense granules in XLA patients was not significantly different than in normal controls. Notably, univariate Cox-regression analysis of dichotomized aggregation responses to low dose collagen in patients on ibrutinib (n=30) revealed a non-significant trend towards increased bleeding risk (HR 1.97, $P=0.135$).

Additionally, we also measured platelet aggregation and ATP release in response to ADP. Both aggregation and ATP release were impaired compared with healthy controls in all patients with CLL, irrespective of treatment status. Mean reduction in aggregation with 10 μM ADP compared to normal controls was 57% for treatment-naïve

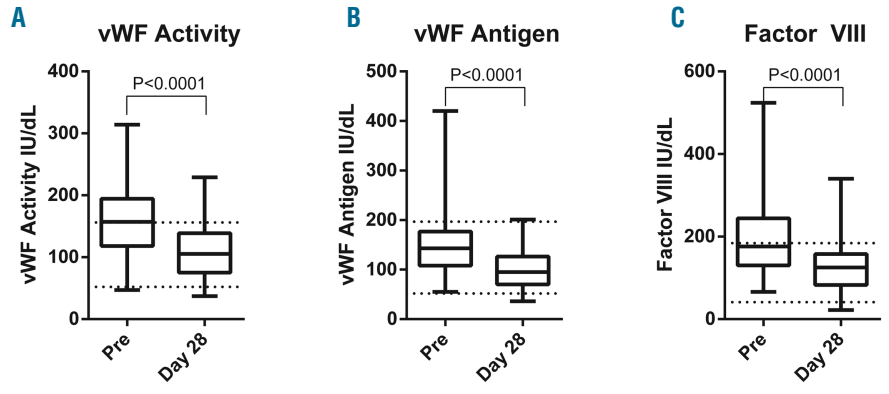

Figure 2. Analysis of vWF activity, vWF antigen and Factor VIII levels in patients on ibrutinib. (A-C) Change in vWF activity, vWF antigen, and Factor VIII levels on day 28 compared to baseline. Dashed lines indicate normal reference range. Comparison by Paired Student's *t*-test.

Table 2. Multivariate analysis of risk factors for bleeding or bruising.

Models*	Variable	N	Adjusted Hazard Ratio (95% CI)	*P*
1	Epi Closure Time, ≥ 240 sec	66	2.74 (1.25 – 5.99)	0.012
2	Factor VIII, ≤ 174 IU/dL	77	3.73 (1.81 – 7.68)	0.0004
3	vWF Activity, ≤ 100 IU/dL	77	2.73 (1.29 – 5.79)	0.009
4	Epi Closure Time, ≥ 240 sec	60	2.55 (1.09 – 5.98)	0.031
	Factor VIII, ≤ 174 IU/dL		2.94 (1.24 – 6.95)	0.014
	vWF Activity, ≤ 100 IU/dL		2.29 (0.99 – 5.32)	0.054

**Age and prior treatment status were included in each of the Cox regression models.*

CLL patients (*P*<0.0001) and 24% (*P*=0.032) for patients on ibrutinib (Figure 4C). Mean reduction in ATP release was 79% (*P*=0.0003) and 43% (*P*=0.039) for these groups, respectively. Unexpectedly, patients on ibrutinib had better aggregation (a 74% increase over CLL, *P*=0.0032) and increased ATP release (a 170% increase over CLL, *P*=0.039) in response to ADP than treatment-naïve CLL patients, suggesting that the CLL-associated platelet dysfunction is improved on treatment with ibrutinib. Responses to ADP were unimpaired in patients with XLA.

Discussion

When we initiated this clinical trial in 2011, early experience with ibrutinib suggested an increased risk of bleeding-related adverse events. We therefore prospectively incorporated clinically relevant platelet function testing and measurements of coagulation factors into the trial design in order to define predictors of, and study the mechanism for, bleeding on ibrutinib. Grade 1-2 bleeding or bruising-related adverse events were recorded in 55% of our patients on ibrutinib over a median follow-up of 24 months, with 51% of all patients experiencing an event within the first 6 months. Notably, the cumulative incidence of bleeding plateaued by 6 months, suggesting that the risk of bleeding on continued therapy decreases. In comparable cohorts of CLL patients receiving alternative therapy, minor bleeding has been reported in between 12% (at a median duration of 5.3 months on ofatumumab) and 17% of patients (at a median duration of 5.5 months on placebo plus rituximab).[11,21] Adjusting for the differences in exposure duration, we estimate that ibrutinib may increase the risk of minor bleeding or bruising by approximately twofold. The bleeding we observed is similar to the mild bleeding diathesis of patients with qualitative platelet defects such as deficiency of GpIa,[22] GpIIb/IIIa and VI,[23,24] that are characterized by easy bruising, epistaxis, gingival bleeding and prolonged bleeding after injury. In contrast, we encountered no bleeding complications associated with lymph node biopsies obtained in the days immediately after starting treatment.

Thrombocytopenia, per se, is unlikely to be the cause for bleeding on ibrutinib since treatment with the drug induced a prompt increase or normalization in platelet counts. Platelet cofactors or coagulation factors such as FVIII and vWF were above normal at baseline in most patients, perhaps as a response to chronic inflammation and CLL related cytokine secretion. The observed normalization in FVIII levels and vWF activity likely reflects an anti-inflammatory effect of ibrutinib, as activation of CLL cells and the secretion of cytokines is inhibited.[1,25] Relatively lower levels of FVIII and vWF activity at baseline, while still within the normal range, were associated with a two- to three-fold increased risk of bleeding-related events (Figure 4). Notably, by day 28 of treatment there were no significant differences in the level of either factor between patients with or without an adverse event. We hypothesize that low normal levels of FVIII and vWF at baseline may denote a group of patients who are less responsive to pro-coagulant inflammatory signals. Conversely, higher baseline FVIII/vWF levels may be protective against bleeding. Collagen present in the subendothelial matrix is a physiologic activator of platelets through a phosphorylation cascade resembling that induced by B-cell receptor activation.[26] BTK, the target of ibrutinib, plays a role in collagen-mediated platelet activation through the GpVI receptor.[12-14,27,28] However, others have found that ibrutinib can impair collagen-induced platelet aggregation.[17,18] In particular, Kamel *et al.* documented a 79% and 52% reduction in platelet aggregation in response to 2 μg/ml and 10 μg/ml collagen, respectively

(but normal responses to 2.5 or 5 μM ADP) in CLL patients pre- and post-ibrutinib therapy.[18] Further, Levade *et al.* reported inhibition of collagen-induced *in vitro* aggregation when ibrutinib was added to normal platelets, and showed that ibrutinib inhibits platelet adhesion onto vWF under high shear rate *in vitro*.[19] The latter finding is consistent with our observation that relatively higher vWF levels may decrease the risk of bleeding (Figure 3). In the same report 14 CLL patients had aggregation studies with collagen at baseline and after 2 to 4 weeks of starting treatment with ibrutinib demonstrated decreased collagen-mediated aggregation on ibrutinib, and decreased *in vitro* binding to vWF under arterial flow. These findings were correlated with the occurrence of four grade ≤2 bleeding events and one grade 3 hemorrhage. Notably, both Kamel *et al.* and Levade *et al.* used platelet rich plasma, rather than whole blood, as we did, so the effects of the CLL cells on platelet aggregation could not be assessed in their studies. Both groups noted a mild baseline decrease in platelet aggregation with collagen in CLL patients as compared to healthy donors prior to initiation of ibrutinib (as did we), which they attributed to thrombocytopenia. ADP aggregation was apparently not significantly affected in either report.[18,19]

To our knowledge, decreased ADP-induced aggregation in CLL patients has only been previously demonstrated in a single case series involving 3 patients.[29] The addition of CLL lymphocytes to platelet rich plasma *in vitro* has been shown to decrease platelet aggregation responses to ADP.[20] Based on these prior findings we believe that diminished platelet aggregation with both collagen and ADP in CLL patients before treatment with ibrutinib is due to the expression of the CD39/NTDPase-1 protein on the surface of CLL cells. CD39/NTDPase-1 has been shown to be present on the surface of ~95% of B cells (normal or malignant).[20] NTDPase-1 inhibits platelet aggregation in part by converting ADP, an important platelet agonist and critical constituent of dense granules that serves to recruit other platelets, upon activation, to AMP. CD39 is expressed on healthy endothelial cells where its chief function is to down-modulate platelet activation at the interface between blood and the blood vessel wall by degrading ADP to AMP. By using whole blood we were able to see the effect of CLL cells on platelet aggregation and dense granule release in response to both collagen and ADP. This is relevant as it reveals an underlying disease-related dysfunction in platelet aggregation that may explain two notable observations; first, ibrutinib considerably improved platelet response to ADP stimulation compared to untreated CLL and second, with continued treatment of the disease, the incidence of new bleeding events decreased.

Because ibrutinib not only inhibits BTK but several additional kinases, we included patients with XLA (congenitally deficient in BTK) as a control group when testing collagen and ADP-stimulated platelet aggregation. Compared to healthy controls, we observed an impairment in collagen (but not ADP) mediated platelet aggregation in XLA patients, consistent with the importance of BTK in collagen-induced signaling in platelets. Notably, aggregation response to collagen was not substantially different in ibrutinib-treated patients than in XLA patients who did not have a bleeding diathesis, suggesting that inhibition of BTK is sufficient to impair platelet response to collagen-mediated aggregation, but that this effect alone cannot account for ibrutinib-related bleeding.

By systematically screening for bleeding-related abnor-

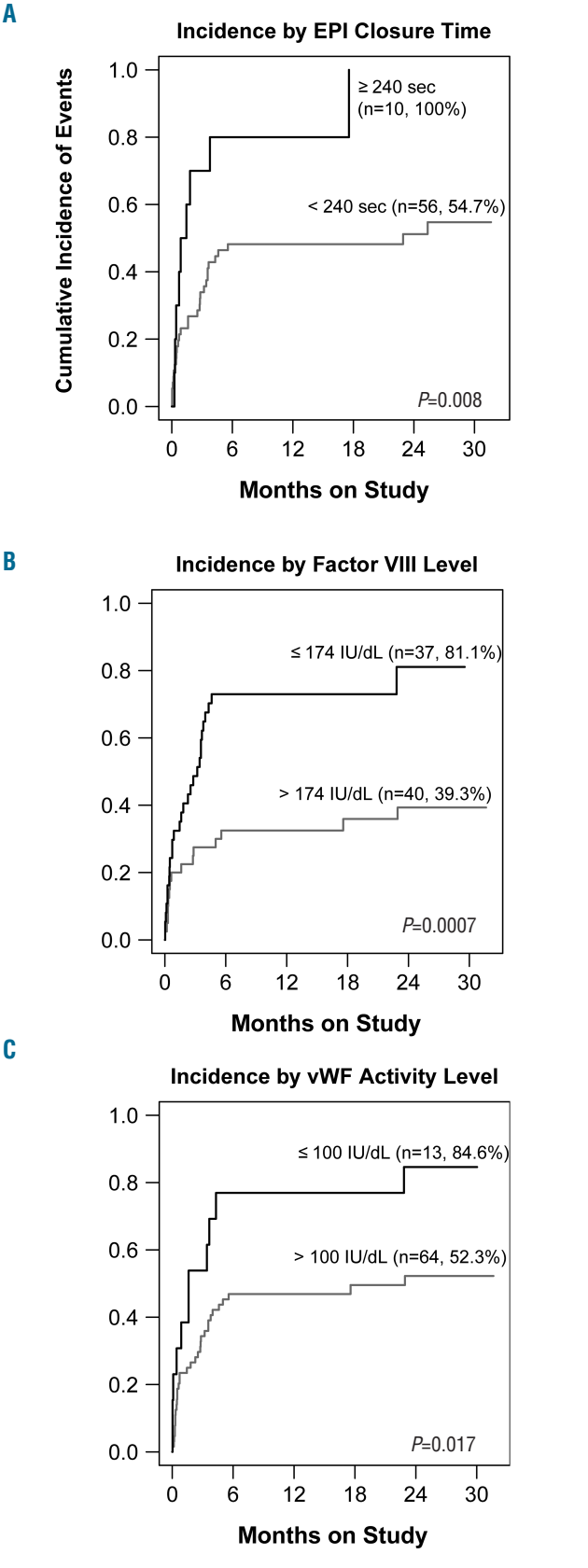

Figure 3. Baseline predictors of an increased risk of developing a bleeding-related adverse event on ibrutinib. (A-C) Cumulative incidence of bleeding stratified by dichotomized cut-offs for EPI closure time, Factor VIII level, and vWF activity. *P* values were calculated via univariate Cox regression analyses.

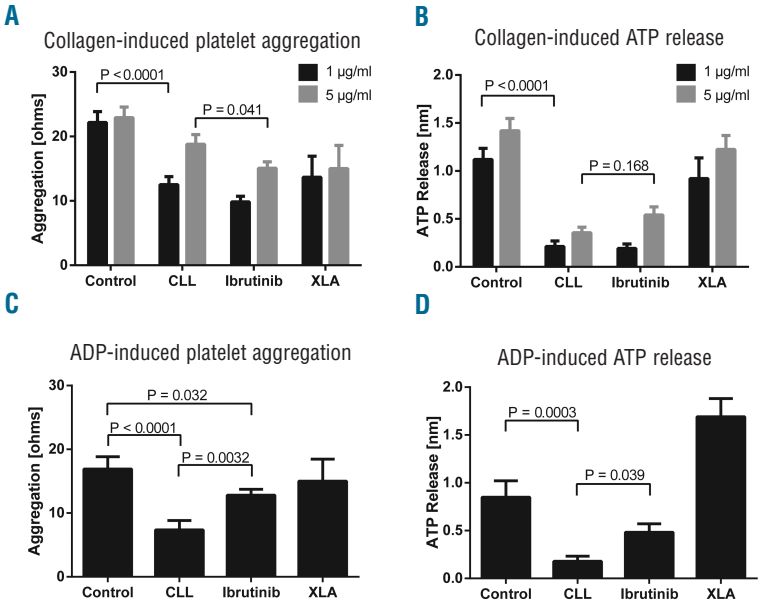

Figure 4. Collagen-induced platelet aggregation and dense granule release. Platelet aggregation and dense granule release in response to collagen in normal controls (n=12), treatment-naïve CLL patients (n=14), CLL patients on ibrutinib (n=30), and XLA patients (n=3). All graphs show means +/- SEM. (A) Maximum 5 µg/mL collagen-induced aggregation at 6 minutes was significantly inhibited in treatment-naïve CLL patients and ibrutinib-treated CLL patients, compared to healthy controls. Results in XLA patients are shown for comparison. Ibrutinib-treated patients exhibited impaired aggregation compared to treatment-naïve CLL patients (mean reduction 19%, *P*=0.041 at high dose). XLA patients demonstrated aggregation in a similar range to patients on ibrutinib at high dose. (B) Dense granule release was decreased by >80% in treatment-naïve CLL patients and by >60% in CLL patients on ibrutinib, but was in the normal range for XLA patients. (*P*<0.0001 between control and CLL patients or ibrutinib patients). (C) Untreated CLL patients exhibited impaired ADP-induced platelet aggregation compared to untreated controls. Platelets from ibrutinib-treated patients exhibited an intermediate level of ADP aggregation that was greater than platelets of untreated CLL patients, yet less robust than healthy controls. (D) Measurement of dense granule release after ADP stimulation demonstrated a marked defect in untreated CLL patients, with platelets from ibrutinib-treated patients also exhibiting a defect compared to healthy controls, but superior ATP release than in untreated CLL patients.

malities in a large group of patients, we detected several aberrations in ibrutinib-treated and treatment-naïve CLL patients. Notably, only baseline platelet function and coagulation factor differences predicted bleeding events in this series, while other variables assessed during treatment were not predictive, suggesting that pre-existing factors determine which patients bleed or bruise on ibrutinib. Specifically, the increased risk associated with prolongation of EPI closure time at baseline may reflect underlying subtle defects in platelet function, or a contribution from drugs or diet that affects platelet function; importantly, the latter is a modifiable risk factor. Given that we observed no serious bleeding complications on ibrutinib, the risk factors identified here were correlated with low grade bleeding events and bruising. Several factors limit our ability to extrapolate the prognostic significance of these variables to predict more severe bleeding. These include the infrequent co-administration of novel oral anticoagulants in our cohort, the exclusion of patients on warfarin, and the relatively younger age of our patients compared to most patients with CLL. Except for concomitant warfarin use, no study has thus far reported on the risk factors in patients with grade ≥3 bleeding events.

In summary we have shown that in *in vitro* tests CLL patients have mild defects of platelet function that may be exacerbated through inhibition of collagen-dependent platelet aggregation by ibrutinib, the use of NSAIDs, and dietary supplements. However, the observed impairment in collagen-induced aggregation on ibrutinib may be partially offset by improvement in ADP-induced collagen aggregation and granule release as the disease improves on continued therapy. Ultimately, the interaction of baseline abnormalities with these ibrutinib-related effects may determine the risk of bleeding in CLL patients on ibrutinib. Notably, the risk of bleeding appears to change with time on treatment and may be highest at the initiation of treatment, when disease-related impairment of collagen and ADP-mediated platelet aggregation and dense granule release, the use of NSAIDs and dietary supplements, and ibrutinib-induced changes in vWF and FVIII as well as further impairment of collagen-mediated platelet aggregation combine. Our data suggest that patients who are at greater risk of clinically significant bleeding can be detected by a few simple clinical laboratory tests and counseled to avoid aspirin, NSAIDs and fish oils that may further predispose them to bleeding. The apparent decrease in bleeding risk on continued therapy reinforces the conclusion that disease-related factors are important contributors to bleeding risk, and suggests that once the disease is controlled, perhaps the judicious introduction of anticoagulants or anti-platelet agents could be considered.

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Authorship and Disclosures

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