THE LANCET

Supplementary appendix

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SUPPLEMENTARY APPENDIX

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SUPPLEMENTAL METHODS

Pharmacokinetics

Pharmacokinetic (PK) parameters were evaluated on Day 1 and Day 8 at different time points following dosing with 100 mg acalabrutinib in 45 relapsed/refractory mantle cell lymphoma patients. Plasma samples for PK analysis were taken predose and at 0.5, 0.75, 1, 2, 4, and 6 hours postdose. On Days 15, 22, and 28 plasma samples were taken at predose and 1-hour postdose administration. Plasma samples were analyzed using a validated liquid chromatography/tandem mass spectrometry assay method with an analytical range of 1.00-1000 ng/mL. Noncompartmental PK analysis of individual plasma acalabrutinib concentration-time data was done using Phoenix® WinNonlin® (version 6.4). Concentration versus time data for Day 1 were calculated with below limit of quantification (BLQ) predose concentrations imputed as zero. Three subjects out of 44 on Day 1 had anomalous initial concentrations that were above BLQ and were included in the mean concentration versus time plot (Figure 1) and PK parameter calculations (Table S3).

Actual sample times were used in the calculation of PK parameters that included maximum concentration (C_{max}), time at which C_{max} occurred (T_{max}), area under the plasma drug concentration-time curve from the time of dosing to the time of the last measurable concentration (AUC_{last}), area under the plasma drug concentration-time curve between times zero and infinity (AUC_{INF}), first order rate constant associated with the terminal (log-linear) portion of the curve (λz), terminal half-life ($t_{1/2}$), apparent total body clearance as a function of bioavailability (CL/F), and apparent volume of distribution as a function of bioavailability (Vz/F).

Bruton Tyrosine Kinase Target Occupancy Enzyme-Linked Immunoassay

Blood for pharmacodynamic analysis was drawn from 36 patients and collected in heparin-coated vacutainer tubes just prior to dosing on days 1, 28, and 56; and 4 hours after dosing on days 1 and 8. Samples were shipped at ambient temperature overnight to Acerta Pharma analytical labs and immediately purified to obtain peripheral blood mononuclear cells (PBMCs) using Ficoll Paque Plus™ density separation method (G.E. Healthcare Biosciences AB; Uppsala, Sweden; Product insert instructions 71-7167-00 AG), followed by cryopreservation in liquid nitrogen. Plasma was cryopreserved for cytokine analysis.

Cryopreserved cells were thawed (37°C water bath), washed in Roswell Park Memorial Institute medium (RPMI) + 1% fetal bovine serum (FBS) RPMI. Five million cells per sample were washed with 1 mL cold phosphate-buffered saline (PBS) and cell pellets were snap-frozen in liquid nitrogen for occupancy assay. Remaining cells were resuspended in RPMI + 10% FBS for signaling assays. Ninety-six-well Optiplate (PerkinElmer; Waltham, MA) plates were coated overnight with 125 ng per well anti-Bruton Tyrosine Kinase (BTK) antibody (BD Biosciences) and blocked with bovine serum albumin. Frozen cell pellets were lysed in ice-cold lysis buffer containing 50 mM Tris-HCl pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% digitonin, and protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO). Cell lysates were incubated for 1 hour on ice in the presence or absence of acalabrutinib (1 µM). At this concentration, BTK was shown in preclinical studies to be saturated and completely bound by acalabrutinib. The cell lysates were incubated for 1 hour on ice with a biotinylated derivative of acalabrutinib (ACP-4016; 10–7 M). The equivalent of 5×10^5 cells of lysate per well, in replicates of 4, were incubated for 2 hours at ambient temperature on the anti-BTK coated 96-well OptiPlate. Plates were washed with PBS + 0.05% Tween four times. Streptavidin-HRP (Invitrogen; enzyme-linked immunoassay [ELISA] grade) was added at 100 µL per well (120 ng/mL) and incubated for 1 hour at room temperature. Plates were washed with PBS + 0.05% Tween 3 times and then washed 2 times with PBS. One hundred microliters per well of SuperSignal ELISA Femto Substrate (ThermoFisher Scientific; Waltham, MA) was added and then chemiluminescence was measured after 1 minute (EnVision® plate reader; PerkinElmer; Waltham, MA). The percent of BTK occupancy for each sample time point was calculated relative to the Day-1 predose sample for each patient. The signal from the day 1 predose sample without exogenous acalabrutinib represents 100% free BTK (or 0% occupied BTK), whereas the signal from the day 1 predose sample with exogenous acalabrutinib represents 0% free BTK (or 100% occupied BTK). The incubation of each cell lysate with 1 µM acalabrutinib was used to correct for background signal not related to free BTK.

$$\% \text{ Free Btk sample X} = \left[\frac{\text{Sample X - Sample X}^{+\text{ACP196 [1uM]}}}{\text{Day1 Predose - Day1 Predose}^{+\text{ACP196 [1uM]}}} \right] \text{X } 100$$

$$\% \text{ Occupied Btk} = 100\% - \% \text{ Free Btk}$$

Only patients having the requisite PBMC numbers and having a signal-to-noise ratio (dynamic range) ≥5 for the Day-1 predose sample were included in the data analysis, because lower dynamic ranges result in high assay variance.

Cytokine Analyses

Plasma from treatment day 1 and 28, collected immediately prior to dosing from 35 patients was tested for cytokine levels by Luminex xMAP® technology using MILLIPLEX® reagents at Eurofins (Cat. #HCYTMAG-60K-PX38, HCP2MAG-62K-PX23, HCD8MAG-15K; EMD Millipore; Billerica, MA). P values were based on Wilcoxon signed-rank test (R software version 3.4.1). An alpha level of 0·05 was used for statistical testing.

Phenotyping of Blood Cell Subsets

Patient blood from baseline and cycle 2 day 28 was sent directly from the clinical site to a Covance central laboratory, which performed immunophenotyping of whole blood by flow cytometry to quantify T, B, and natural killer (NK) cells. Significance was determined using a paired, 2-tailed, parametric t-test. An alpha level of 0·05 was used for statistical testing.

Lymphocytosis

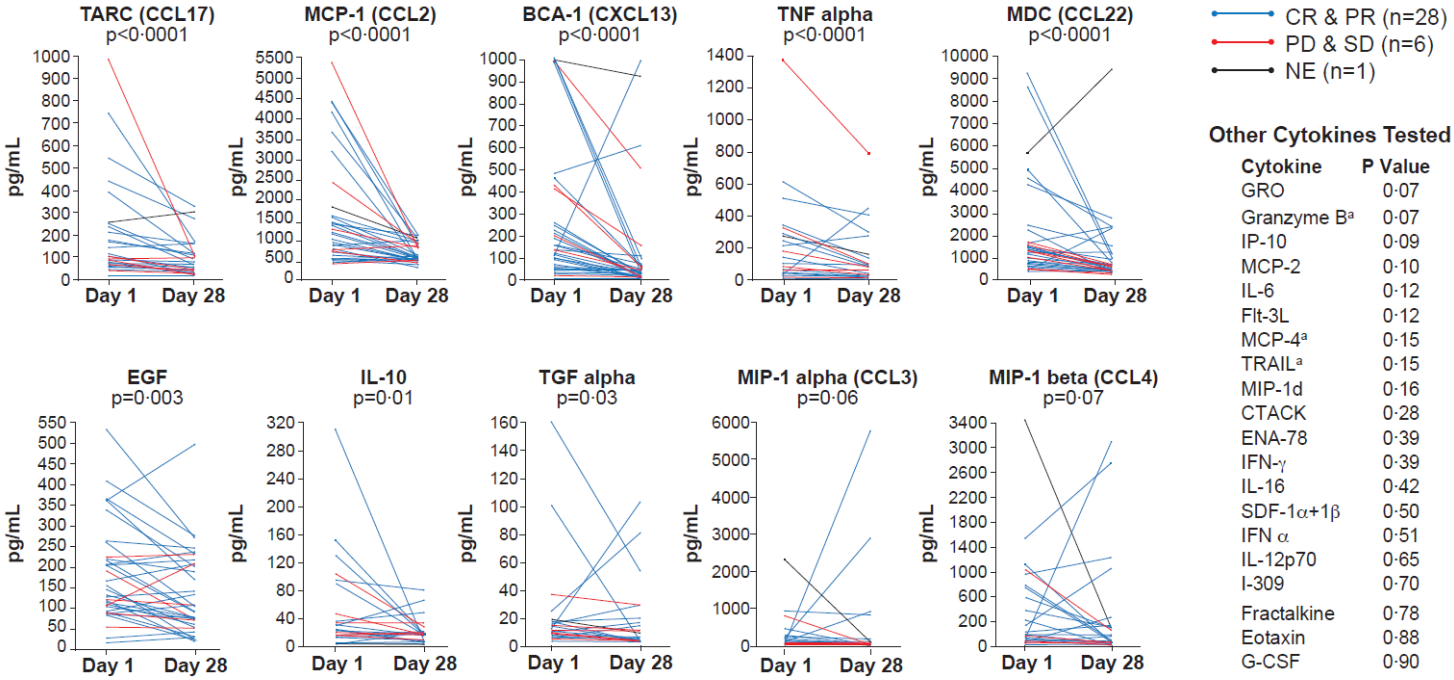
Lymphocytosis was defined as absolute lymphocyte count (ALC) increased ≥50% from baseline and a postbaseline assessment $\geq 5\times10^9$/L. Resolution of lymphocytosis was defined as when ALC decreased to baseline or lower or achieved $<5\times10^9$/L.

SUPPLEMENTAL RESULTS

Pharmacokinetics

Exposure to acalabrutinib was generally variable and there was no major difference in PK parameters due to prior treatment. Acalabrutinib was relatively rapidly absorbed with median plasma T_{max} of 1.04 and 0.77 hr on days 1 and 8, respectively (Table S3). Geometric mean C_{max} were 523 (120%) and 599 (96.8%) ng/mL on days 1 and 8, respectively. Geometric mean acalabrutinib AUC_{last} were 751 (74.1%) and 818 (59.4%) ng/mL on days 1 and 8, respectively. Exposure to acalabrutinib was comparable on days 1 and 8 and predose concentrations on days 8, 15, 22 and cycle 2 day 1 (12 hours after the respective prior dose) were generally less than 5 ng/mL, indicating low potential for accumulation. Acalabrutinib plasma concentrations measured at 1-hour postdose on days 1, 8, 15, 22 and cycle 2 day 1 (12 hours after the respective prior dose) were variable and relatively constant over time, indicating neither accumulation nor induction of acalabrutinib clearance (data not shown). Acalabrutinib was relatively rapidly eliminated, with mean half-lives of 1.15 (63.2%) and 1.05 (42.2%) hours on days 1 and 8, respectively. Acalabrutinib mean calculated apparent oral clearance (CL/F) values were 124 and 128 L/hr and acalabrutinib mean calculated apparent volume of distribution (Vz/F) values were 196 and 206 L on days 1 and 8, respectively. Overall, acalabrutinib PK parameters measured in this study were variable and indicated relatively rapid absorption and elimination and low potential for accumulation.

Figure S1. Plasma Cytokines Before and After Acalabrutinib Treatment (Day 28)

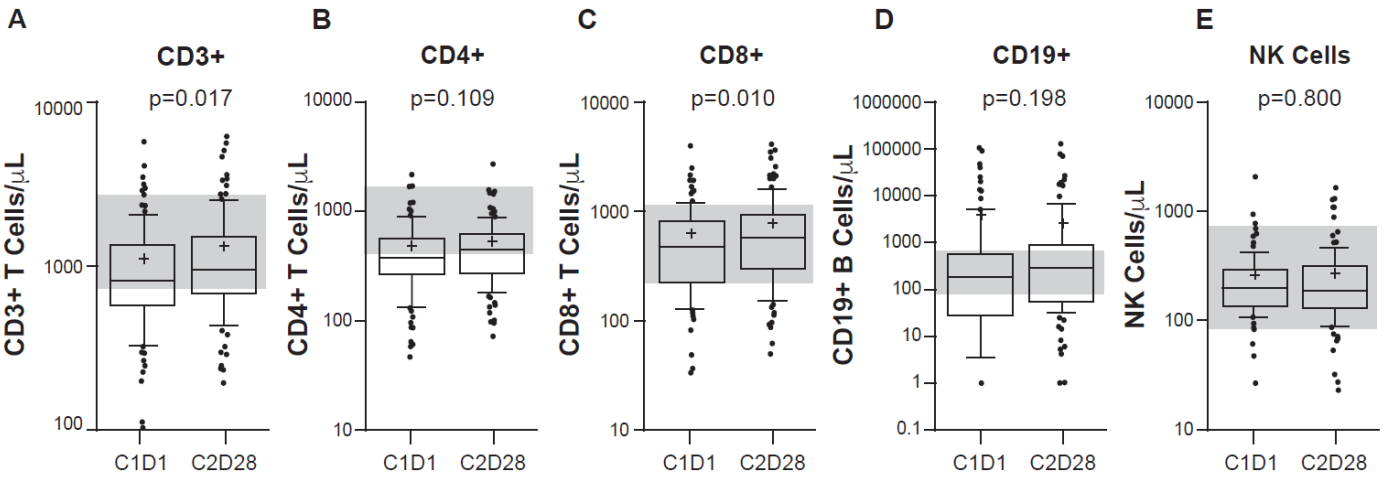


Other Cytokines Tested

Cytokine	P Value
GRO	0·07
Granzyme B[a]	0·07
IP-10	0·09
MCP-2	0·10
IL-6	0·12
Flt-3L	0·12
MCP-4[a]	0·15
TRAIL[a]	0·15
MIP-1d	0·16
CTACK	0·28
ENA-78	0·39
IFN-γ	0·39
IL-16	0·42
SDF-1α+1β	0·50
IFN α	0·51
IL-12p70	0·65
I-309	0·70
Fractalkine	0·78
Eotaxin	0·88
G-CSF	0·90

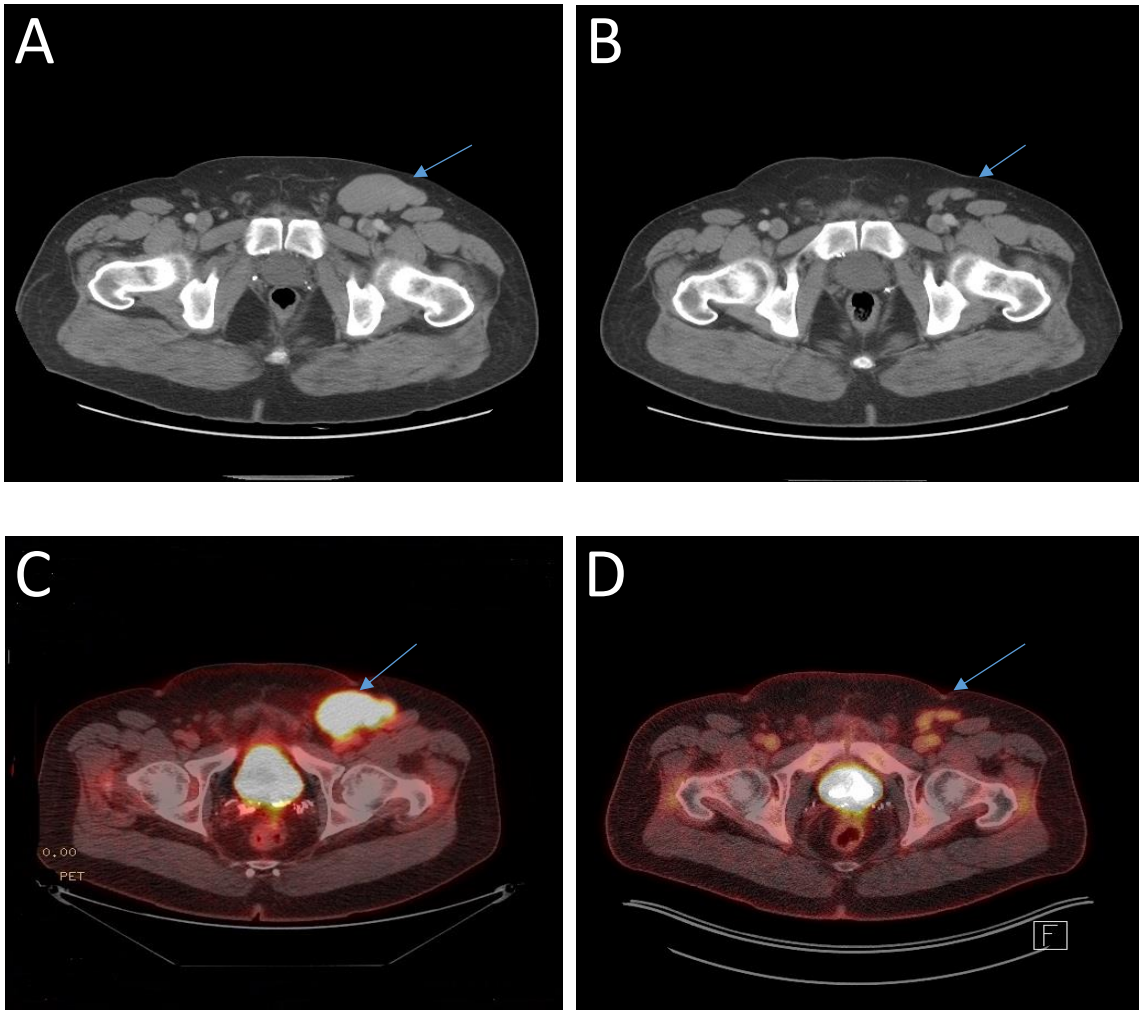
Measurement of cytokines from plasma for 35 patients at baseline (Day 1 prior to treatment) and after 28 days of acalabrutinib treatment. Colors denote best investigator-assessed clinical response according to the Lugano classification. The p values were determined using a paired Wilcoxon test; for significance of change from baseline (Day 1). [a]Granzyme B, n=33; MCP-4, n=34; TRAIL, n=13. CR=complete response. PR=partial response. PD=progressive disease. SD=stable disease. NE=not evaluable.

Figure S2. Blood Cell Subsets in Peripheral Blood



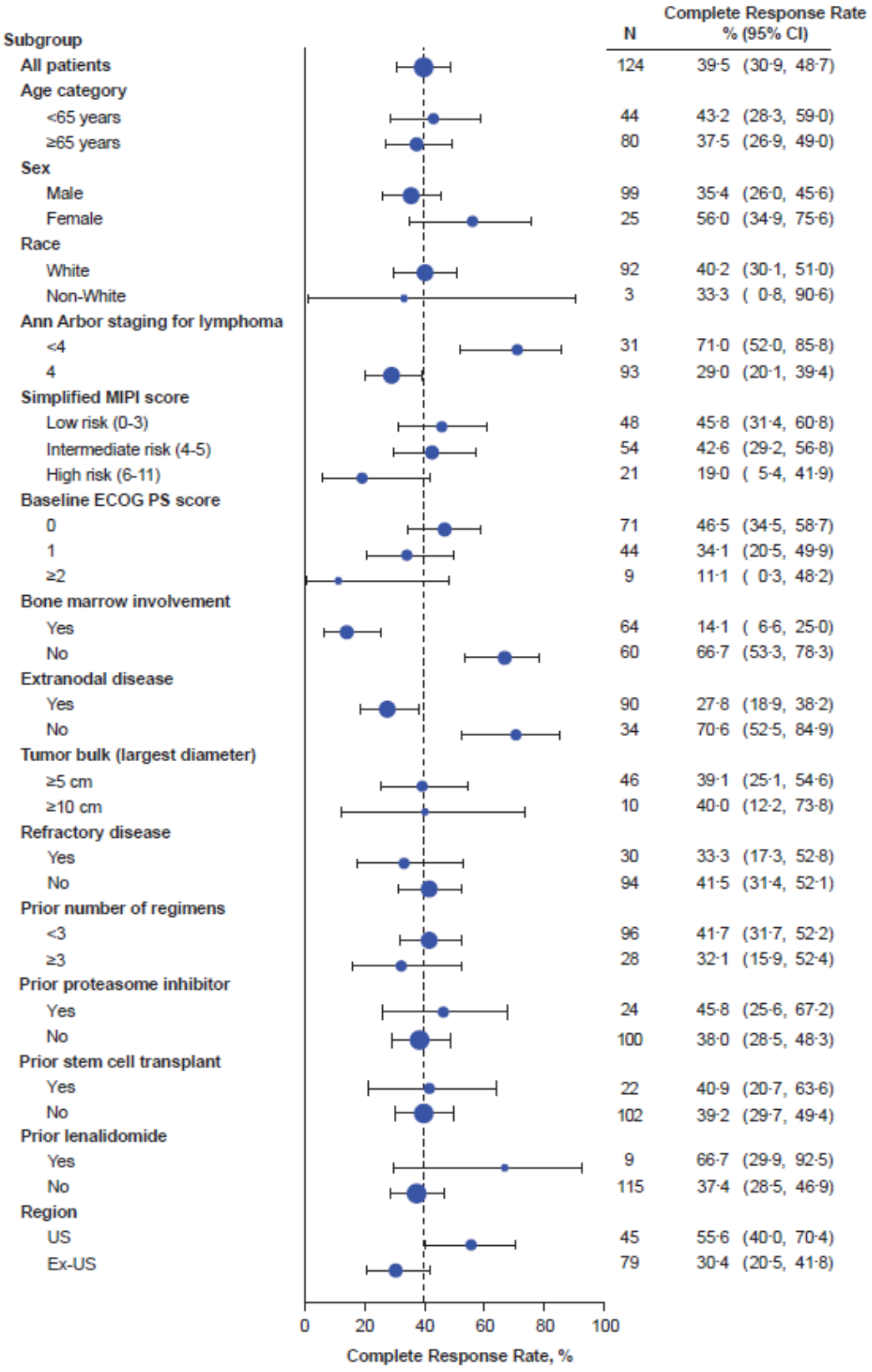
Measurement of blood cell populations before and after 56 days (C2D28) on acalabrutinib treatment (n=109). Panels show total T cells (CD3+) (**A**), CD4+ T-cell subset (**B**), CD8+ T-cell subset (**C**), CD19+ B cells (**D**), and CD16+CD56+ NK cells (**E**). The normal reference range is indicated by the gray area. Whiskers in (**A-C, E**) indicate 10-90 percentiles and whiskers in (**D**) indicate 20-90 percentiles. Plus signs indicate mean averages. Values of zero in (**D**) are not shown. Significance was determined using a paired, 2-tailed, parametric t-test. C=cycle; D=day; NK=natural killer. ns=not significant.

Figure S3. Representative PET/CT Scans of Tumor Response Before and After 2 Cycles of Acalabrutinib



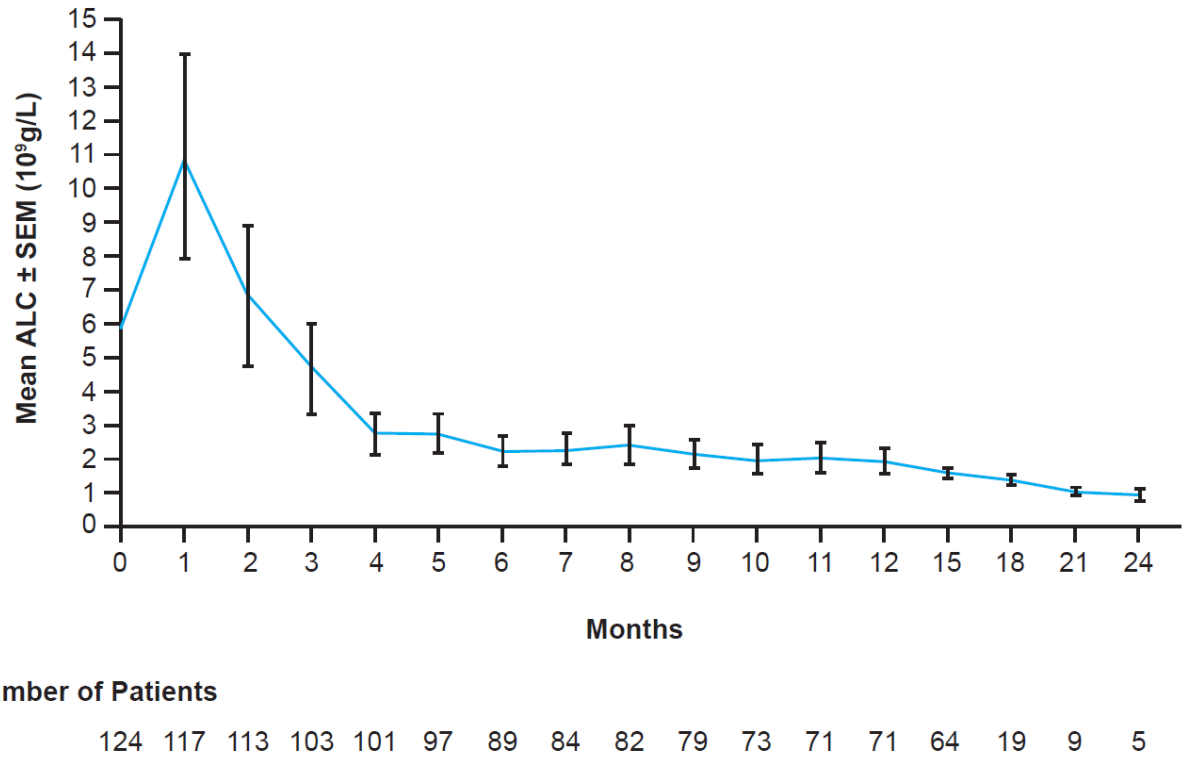
CT (top row) and PET scans (bottom row) of left pelvic mass measuring 7.1 × 3.9 cm before (**A, C**) and 2.1 x 1 cm plus 2.2 x 1.1 cm after (**B, D**) 2 cycles of acalabrutinib. Arrows indicate pelvic mass. CT=computed tomography. PET=positron emission tomography.

Figure S4. Subgroup Analysis of Complete Response Rate



Forest plot containing complete response rate analyzed by prespecified subgroups according to baseline demographic and clinical characteristics. The 95% confidence interval was based on exact binomial distribution. CR=complete response. ECOG PS=Eastern Cooperative Oncology Group Performance Status. MIPI=Mantle Cell Lymphoma International Prognostic Index. US=United States.

Figure S5. Mean Absolute Lymphocyte Counts Over Time in Patients Treated With Acalabrutinib

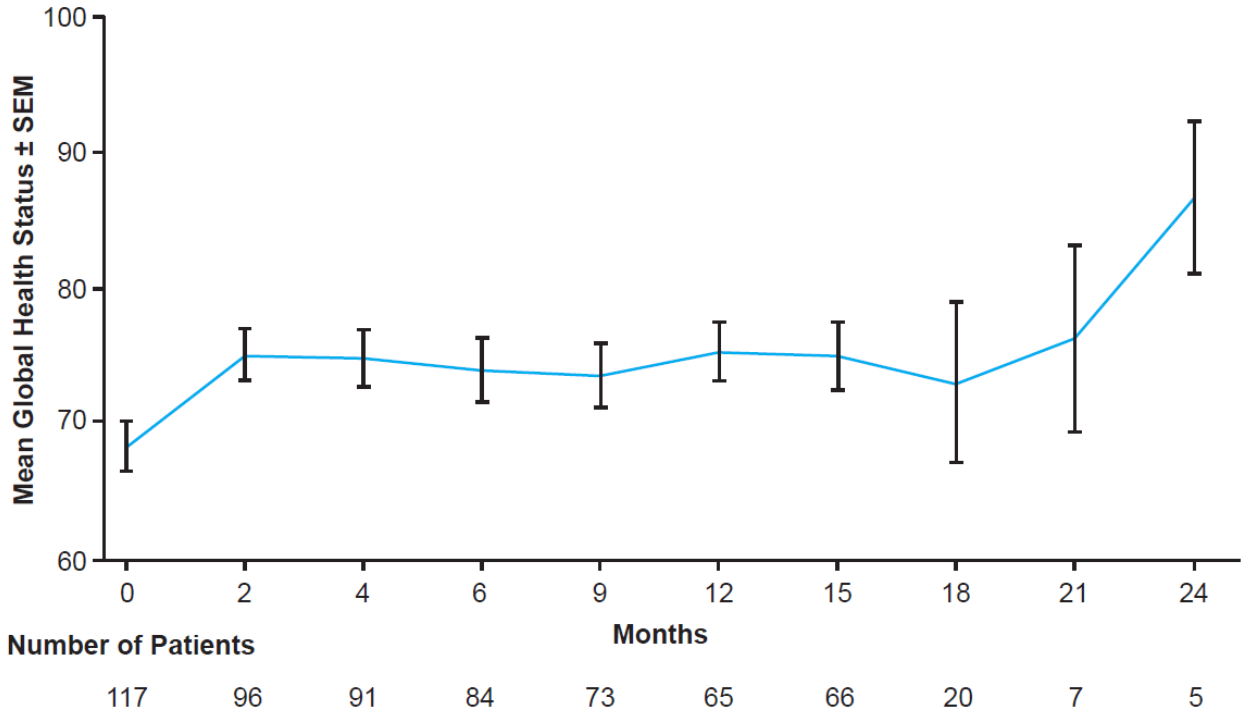


Number of Patients

124	117	113	103	101	97	89	84	82	79	73	71	71	64	19	9	5

ALC=absolute lymphocyte counts. SEM=standard error of the mean.

Figure S6. Mean plot of EORTC QLQ-C30 over time: global health status/quality of life



EORTC=European Organisation for Research and Treatment of Cancer. QLQ-C30=Core Quality of Life Questionnaire. SEM=standard error of the mean.

Table S1. Dose Modification Schedule

AEs requiring dose modification	
• Grade 4 ANC (<500/μL) for >7 days (neutrophil growth factors permitted per ASCO guidelines[1]; use must be recorded on the eCRF) • Grade 3 platelet decreases in the presence of significant bleeding • Grade 4 platelet decreases • Grade 3 or 4 nausea, vomiting, or diarrhea, if persistent despite optimal antiemetic and/or antidiarrheal therapy • Any other Grade 4 toxicity or unmanageable Grade 3 toxicity	
Occurrence	Action
1st-2nd	Hold acalabrutinib until recovery to Grade ≤1 or baseline; may restart at original dose level (100 mg bid)
3rd	Hold acalabrutinib until recovery to Grade ≤1 or baseline; restart at 100 mg qd
4th	Discontinue acalabrutinib

AE=adverse event. ANC=absolute neutrophil count. ASCO=American Society of Clinical Oncology. bid=twice daily. eCRF=electronic case report form. qd=once daily.

Table S2. Adverse Events Leading to Treatment Discontinuation

	N=124
Patients with adverse event leading to treatment discontinuation	7 (6%)
Aortic stenosis	1 (1%)
Diffuse large B-cell lymphoma	1 (1%)
Blood blister[a]	1 (1%)
Petechiae[a]	1 (1%)
Dyspnea[b]	1 (1%)
Leukostasis syndrome[b]	1 (1%)
Noncardiac chest pain	1 (1%)
Pulmonary fibrosis	1 (1%)
Thrombocytopenia	1 (1%)

Data are n (%). [a]Blood blister and petechiae were both observed in 1 patient with Grade 3 acute coronary syndrome which was treated with clopidrogel, resulting in blood blister/petechiae formation (considered treatment-related). [b]Dyspnea and leukostasis syndrome were both observed in 1 patient.

Table S3. Plasma Acalabrutinib Pharmacokinetic Parameter Summary

Variable	Day 1	Day 8
Geometric mean		
C_{max} (ng/mL)	523 (120%), 42	599 (96.8%), 37
AUC_{last} (hr*ng/mL)	751 (74.1%), 44	818 (59.4%), 39
AUC_{INF} (hr*ng/mL)	923 (55.3%), 34	886 (54.1%), 37
Arithmetic mean		
T_{max} (hr)	1.04 (0.5, 4), 42	0.77 (0.4, 2), 37
C_{max} (ng/mL)	715 ± 469 (65.6%), 42	797 ± 576 (72.3%), 37
CL/F (L/hr)	124 ± 71.9 (57.8%), 34	128 ± 66.6(52.2%), 37
Vz/F (L)	196 ± 130 (66.4%), 34	206 ± 159 (77.4%), 37
$t_{1/2}$ (hr)	1.15 ± 0.73 (63.2%), 34	1.05 ± 0.43 (42.2%), 37

Arithmetic mean values are arithmetic mean ± standard deviation (CV%), n except T_{max} values are reported as Median (Min, Max), n. Geometric mean values are geometric mean (geometric mean CV%), n. AUC_{INF}=area under the plasma drug concentration-time curve between times zero and infinity. AUC_{last}=area under the plasma drug concentration-time curve from the time of dosing to the time of the last measurable concentration. C_{max}=maximum concentration. CL/F=apparent total body clearance as a function of bioavailability. T_{max}=time at which C_{max} occurred. $t_{1/2}$=terminal half-life. Vz/F=and apparent volume of distribution as a function of
bioavailability.

Table S4. Response Based on Independent Review Committee Assessment According to the 2007 International Harmonization Project Criteria[2]

	All treated patients (N=124)	
		95% CI[a]
ORR (CR + PR)	93 (75%)	66%, 82%
Best response		
CR	37 (30%)	22%, 39%
PR	56 (45%)	36%, 54%
SD	14 (11%)	6%, 18%
PD	10 (8%)	4%, 14%
Not evaluable[b]	5 (4%)	1%, 9%
No evidence of disease	1 (1%)	0%, 4%
Unknown	1 (1%)	0%, 4%
Median DOR	NR	14·8%, NR

Data are n (%), median, or 95% CI. CI=confidence interval. CR=complete response. ORR=overall response rate. PD=progressive disease. PR=partial response. SD=stable disease. [a]95% exact binomial CI. [b]Includes baseline only patients.

Table S5. Serious Adverse Events Occurring in ≥2 Patients

	N=124
Patients with a serious adverse event	48 (39%)
Pneumonia	5 (4%)
Anemia	4 (3%)
General physical health deterioration	3 (2%)
Sepsis	2 (2%)
Tumor lysis syndrome	2 (2%)
Vomiting	2 (2%)

Data are n (%).

Table S6. Adverse Events of Clinical Interest

Adverse event	All treated patients (N=124)	
	All Grades	Grade 3/4
Infections	66 (53%)	16 (13%)
Pneumonia	7 (6%)	6 (5%)
Hemorrhage	39 (31%)	1 (1%)
Leukopenia	16 (13%)	16 (13%)
Neutropenia	16 (13%)	16 (13%)
Other Leukopenia	1 (1%)	1 (1%)
Anemia	15 (12%)	11 (9%)
Cardiac events	10 (8%)	3 (2%)
Atrial fibrillation	0	0
Acute coronary syndrome[a]	1 (1%)	1 (1%)
Acute myocardial infarction[b]	1 (1%)	1 (1%)
Cardio-respiratory arrest[c]	1 (1%)	1 (1%)
Second primary malignancies	8 (6%)	4 (3%)
Second primary malignancies, excluding skin	4 (3%)	4 (3%)
Thrombocytopenia	7 (6%)	6 (5%)
Hepatic events	5 (4%)	2 (2%)
Hypertension	3 (2%)	1 (1%)
Tumor lysis syndrome	3 (2%)	3 (2%)
Interstitial lung disease/Pneumonitis	2 (2%)	1 (1%)

Data are n (%). SAE=serious adverse event. [a]One patient developed a Grade 3 SAE of acute coronary syndrome on Day 3 that led to dose delay; the event resolved on Day 14. The patient also experienced Grade 2 petechiae on Day 24 that was considered related to study treatment and led to study treatment discontinuation. The patient had a history of stroke and was on prophylactic anticoagulant therapy. [b]One patient developed a Grade 3 SAE of acute myocardial infarction on Day 6 that was considered not related to study treatment and resolved on Day 9. Treatment was held during this time. [c]One patient discontinued study treatment on Day 451 due to disease progression and developed a Grade 4 SAE of cardiorespiratory arrest on Day 463. The patient died on the same day.

Table S7. Infections Occurring in ≥2 Patients

Adverse event	All treated patients (N=124)	
	All Grades	Grade 3/4
Infections	66 (53%)	16 (13%)
Sinusitis	12 (10%)	0
Nasopharyngitis	8 (6%)	0
Upper respiratory tract infection	8 (6%)	0
Bronchitis	7 (6%)	0
Pneumonia	7 (6%)	6 (5%)
Urinary tract infection	5 (4%)	2 (2%)
Conjunctivitis	4 (3%)	0
Lower respiratory tract infection	4 (3%)	0
Herpes zoster	3 (2%)	0
Influenza	3 (2%)	0
Laryngitis	3 (2%)	0
Lung infection	3 (2%)	0
Pharyngitis	3 (2%)	1 (1%)
Respiratory tract infection	3 (2%)	1 (1%)
Cellulitis	2 (2%)	0
Eye infection	2 (2%)	0
Oral herpes	2 (2%)	0
Rhinitis	2 (2%)	0
Sepsis	2 (2%)	2 (2%)
Tracheitis	2 (2%)	0

Data are n (%).

Table S8. Hemorrhage Events

Adverse event	All treated patients (N=124)	
	All Grades	Grade 3/4
Hemorrhage	39 (31%)	1 (1%)
Contusion	16 (13%)	0
Petechiae	11 (9%)	0
Epistaxis	8 (6%)	0
Hematoma	6 (5%)	0
Purpura	5 (4%)	0
Ecchymosis	4 (3%)	0
Increased tendency to bruise	3 (2%)	0
Blood blister	1 (1%)	0
Conjunctival hemorrhage	1 (1%)	0
Gastrointestinal hemorrhage[a]	1 (1%)	1 (1%)
Hematochezia	1 (1%)	0
Hematuria	1 (1%)	0
Rectal hemorrhage	1 (1%)	0
Vessel puncture site hematoma	1 (1%)	0

Data are n (%). [a]Serious adverse event; not considered treatment-related.

References

1. Smith TJ, Bohlke K, Lyman GH, et al. Recommendations for the Use of WBC Growth Factors: American Society of Clinical Oncology Clinical Practice Guideline Update. J Clin Oncol 2015; 33: 3199–212.
2. Cheson BD, Pfistner B, Juweid ME, et al. Revised response criteria for malignant lymphoma. J Clin Oncol 2007; 25:579–86.