SUPPLEMENTARY APPENDIX

The sympathomimetic agonist mirabegron did not lower *JAK2*-V617F allele burden, but restored nestin-positive cells and reduced reticulin fibrosis in patients with myeloproliferative neoplasms: results of phase II study SAKK 33/14

Beatrice Drexler,[1] Jakob R. Passweg,[1] Alexandar Tzankov,[2] Martin Bigler,[3] Alexandre PA Theocharides,[4] Nathan Cantoni,[5] Peter Keller,[6] Georg Stussi,[7] Axel Ruefer,[8] Rudolf Benz,[9] Geneviève Favre,[10] Pontus Lundberg,[1] Ronny Nienhold,[11] Andrea Fuhrer,[3] Christine Biaggi,[3] Markus G. Manz,[4] Mario Bargetzi,[5] Simon Mendez-Ferrer,[12] and Radek C. Skoda;[11] on behalf of the Swiss Group for Clinical Cancer Research (SAKK)

[1]Division of Hematology, University Hospital Basel and University of Basel, Switzerland; [2]Institute of Pathology, University Hospital Basel and University of Basel, Switzerland; [3]Swiss Group for Clinical Cancer Research, Bern, Switzerland; [4]Hematology and Oncology, University Hospital Zurich and University of Zurich, Switzerland; [5]Oncology, Hematology & Transfusion Medicine, Kantonsspital Aarau AG, Switzerland; [6]University Clinic of Hematology and Central Hematology Laboratory, University Hospital Bern, Switzerland; [7]Clinic of Hematology, Oncology Institute of Southern Switzerland, Bellinzona, Switzerland; [8]Departement Medizin, Luzerner Kantonsspital, Switzerland; [9]Kantonsspital Muensterlingen, Switzerland; [10]Cantonal Hospital Liestal, Switzerland; [11]Department of Biomedicine, Experimental Hematology, University Hospital Basel and University of Basel, Switzerland and [12]Wellcome Trust-Medical Research Council Cambridge Stem Cell Institute, Department of Haematology, University of Cambridge, and National Health Service Blood and Transplant, Cambridge, UK

 doi:10.3324/haematol.2018.200014

Received: June 20, 2018.

Accepted: November 8, 2018.

Pre-published: November 8, 2018.

Correspondence: *RADEK SKODA*

radek.skoda@unibas.ch

JAKOB PASSWEG

jakob.passweg@usb.ch

SUPPLEMENTAL METHODS

Inclusion criteria

- Histologically or cytologically confirmed diagnosis of JAK2-V617F positive ET, PV or PMF at primary diagnosis or pretreated
- JAK2-V617F mutant allele burden > 20% in the peripheral blood at study entry
- Patient must give written informed consent before registration
- WHO performance status 0-2 (see Appendix 2)
- Age ≥ 18 years
- Adequate hematological values: neutrophils ≥ 1.5 x 10^9/L, platelets ≥ 100 x 10^9/ L
- Adequate hepatic function: bilirubin ≤ 1.5 x ULN, AST/ALT/AP ≤ 2.5 x ULN
- Adequate renal function (calculated creatinine clearance > 50 mL/min, according to the formula of Cockcroft-Gault, see Appendix 3)
- Women are not breastfeeding. Women with child-bearing potential are using effective contraception (see section 9.5), are not pregnant and agree not to become pregnant during participation in the trial and during 28 days thereafter. A negative pregnancy test before inclusion (within 7 days) into the trial is required for all women with child-bearing potential. Men agree not to father a child during participation in the trial and during 28 days thereafter.
- Patient compliance and geographic proximity allow proper staging and follow-up.

Exclusion criteria

- Leukemic transformation (>20% blasts in blood, marrow or extramedullary site)
- Diabetic neuropathy
- Severe or uncontrolled cardiovascular disease (congestive heart failure NYHA III or IV (see Appendix 4), unstable angina pectoris, history of myocardial infarction within the last twelve months, known cardiac rhythm disturbance including atrial fibrillation or QT prolongation (see Appendix 5)
- Uncontrolled hypertension
- Treatment of ET, PV or PMF with IFNα or treatment of PMF with JAK inhibitors such as ruxolitinib within 3 months prior to trial entry.
- Previous malignancy within 5 years with the exception of adequately treated cervical carcinoma in situ or localized non-melanoma skin cancer.
- Psychiatric disorder precluding understanding of information on trial related topics, giving informed consent or interfering with compliance for oral drug intake.
- Treatment with hematopoietic stem cell transplantation
- Concurrent treatment with cytoreductive drugs (see section 9.2.4), other experimental drugs or other anti-cancer therapy as well as treatment in a clinical trial within 2 months prior to trial entry.

- Any serious underlying medical condition (at the judgment of the investigator), which could impair the ability of the patient to participate in the trial (e.g. active autoimmune disease, uncontrolled diabetes, uncontrolled infection, HIV, Hepatitis B and C).
- Known hypersensitivity to trial drug or hypersensitivity to any other component of the trial drug.
- Any concomitant drugs contraindicated for use with the trial drug according to the approved product information.

Additional details on the study design

In patients with adverse effects attributed to mirabegron, dose reduction to 25 mg was allowed if the prior dose was 50 mg. Severe adverse events ≥ G3 by common toxicity criteria that were at least possibly related to trial treatment led to discontinuation of the study drug. After completion of the 24 weeks treatment, patients with an allele burden lower than at study entry and patients with a reduction in allele burden of at least 25% at 12 weeks compared to study entry had the option to stay on treatment for maximally 2 additional years, or until their allele burden reached baseline levels or higher. All other patients were directly transferred to the follow-up phase after 24 weeks.

Secondary Endpoints

Secondary endpoints were: reduction of the *JAK2*-V617F allele burden ≥ 50% at 12 weeks, reduction of the *JAK2*-V617F allele burden ≥ 25% at 12 weeks or 24 weeks, reduction of disease related symptoms, duration of response, adverse events, and overall hematological response using criteria defined by the European LeukemiaNet (ELN) and International Working Group-Myeloproliferative Neoplasms Research and Treatment (IWG-MRT), explained in detail below.[1,2]

Disease-related symptoms

System organ class	Disease symptom
Skin and subcutaneous tissue disorders	Pruritus
General disorders and administration site conditions	Fatigue
General disorders and administration site conditions	Fever
Gastrointestinal disorders	Abdominal distension
Gastrointestinal disorders	Early satiety
Nervous system disorders	Headache
Microvascular	Microvascular disturbance: • erythromelalgia • acroparesthesia • digital ischemia

Response criteria for ET and PV (according to the European LeukemiaNet (ELN) and International Working Group-Myeloproliferative Neoplasms Research and Treatment (IWG-MRT).[1]

Definition		
	Essential thrombocythemia	**Polycythemia vera**
Complete response	(1) Platelet count ≤ 400 x 109/L , AND (2) no disease-related symptoms,* AND (3) normal spleen size on imaging, AND (4) white blood cell count ≤ 10 x 109/L	(1) Hematocrit < 45% without phlebotomy AND (2) platelet count ≤ 400 x109/L AND (3) white blood cell count ≤ 10 x 109/L, AND (4) normal spleen size on imaging AND (5) no disease-related symptoms*
Partial response	In patients who do not fulfill the criteria for complete response, platelet count ≤ 600 x 109/L OR decrease > 50% from baseline	In patients who do not fulfill the criteria for complete response, hematocrit < 45% without phlebotomy OR response in 3 or more of the other criteria
No response	Any response that does not satisfy partial or complete response	Any response that does not satisfy partial or complete response

*Disease-related symptoms: see table disease-related symptoms.

Response criteria for PMF (according to ELN and IWG-MRT).[2]

	Primary Myelofibrosis
Complete remission	Bone marrow:* Age-adjusted normocellularity; <5% blasts; ≤ grade 1 MF† and Peripheral blood: Hemoglobin ≥ 100 g/L and < ULN; neutrophil count ≥ 1 x 10^9/L and < ULN; Platelet count ≥ 100 x 10^9/L and < ULN; < 2% immature myeloid cells‡ and Clinical: Resolution of disease symptoms; spleen and liver not palpable; no evidence of EMH
Partial remission	Peripheral blood: Hemoglobin ≥ 100 g/L and < ULN; neutrophil count ≥ 1 x 10^9/L and < ULN; Platelet count ≥ 100 x 10^9/L and < ULN; < 2% immature myeloid cells‡ and Clinical: Resolution of disease symptoms; spleen and liver not palpable; no evidence of EMH Bone marrow:* Age-adjusted normocellularity; <5% blasts; ≤ grade 1 MF†, and peripheral blood: Hemoglobin ≥ 85 but < 100 g/L and <UNL; neutrophil count ≥ 1x 10^9/L and <UNL; platelet count ≥50, but < 100 x 10^9/L and <UNL; < 2% immature myeloid cells‡ and Clinical: Resolution of disease symptoms; spleen and liver not palpable; no evidence of EMH
Anemia response	Transfusion-independent patients: a ≥ 20 g/L increase in hemoglobin level‖ Transfusion-dependent patients: becoming transfusion-independent{
Spleen response#	A baseline splenomegaly that is palpable at 5-10 cm, below the LCM, becomes not palpable** or A baseline splenomegaly that is palpable at > 10 cm, below the LCM, decreases by ≥ 50%** A baseline splenomegaly that is palpable at < 5 cm, below the LCM, is not eligible for spleen response A spleen response requires confirmation by sonography showing ≥ 35% spleen volume reduction
No response	Any response that does not satisfy complete/partial remission or anemia or spleen response.

EMH: extramedullary hematopoiesis (no evidence of EMH implies the absence of pathology- or imaging study-proven non-hepatosplenic EMH); LCM, left costal margin; ULN, upper limit of normal.
*Baseline and posttreatment bone marrow slides are to be interpreted at one sitting by a central review process. Cytogenetic and molecular responses are not required for CR assignment.
†Grading of MF is according to the European classification
‡ Immature myeloid cells constitute blasts, promyelocytes, myelocytes, metamyelocytes and nucleated red blood cells. In splenectomized patients, 5% immature myeloid cells are allowed.
‖ Applicable only to patients with baseline hemoglobin of < 100 g/L. In patients not meeting the strict criteria for transfusion dependency at the time of study enrollment (see as follows), but having received transfusions within the previous month, the pre-transfusion hemoglobin level should be used as the baseline.
{ Transfusion dependency before study enrollment is defined as transfusions of at least 6 units of packed red blood cells (PRBC), in the 12 weeks prior to study enrollment, for a hemoglobin level of < 85 g/L, in the absence of bleeding or treatment-induced anemia. In addition, the most recent transfusion episode must have occurred in the 28 days prior to study enrollment. Response in transfusion-dependent patients requires absence of any PRBC transfusions during any consecutive "rolling" 12-week interval during the treatment phase, capped by a hemoglobin level of ≥ 85 g/L.
In splenectomized patients, palpable hepatomegaly is substituted with the same measurement strategy.
** Spleen or liver responses must be confirmed by imaging studies where a ≥ 35% reduction in spleen volume, as assessed by ultrasound, is required. Furthermore, a ≥ 35% volume reduction in the spleen or liver, by ultrasound, constitutes a response regardless of what is reported with physical examination.

Library preparation and target region capture for next generation sequencing

A total of 500 ng of granulocyte DNA derived from peripheral blood of each patient was fragmented using Covaris E220 Focused-ultrasonicator (Covaris Inc.), resulting in an average fragment size of ≈250. The fragmented library was purified using Agencourt AMPure XP beads (Beckman Coulter Inc.). Following purification, the library was end-repaired, adenylated and adapters were ligated using the NEXTflex Rapid DNA-Seq Kit and NEXTflex-96™ DNA Barcodes (Bio Scientific). Subsequently, adaptor-ligated DNA, each assigned with a different barcode, was pooled equimolarly in duplicate tubes.

Bait design and target capture

Capture of target regions was performed using an Agilent SureSelect custom design including the targeted exons ± 50 bp of flanking regions with a total size of ≈ 0.44 Mb. Enrichment was performed using the provided Agilent protocol and capture was performed for 72 hours. Postenrichment polymerase chain reaction (PCR) was performed for 10 cycles.

Next generation sequencing and data analysis

Paired-end 100-bp cycle sequencing of the captured libraries was performed using an Illumina HiSeq2500. Demultiplexed samples were mapped and analyzed using the CLC genomics workbench. Mapping was performed using a mismatch cost of 2 and insertion and deletion cost of 3 with a length fraction 0.7 and similarity fraction of 0.8. For mutational calling, the quality-based variant detection was used, using a neighborhood radius of 5, maximum gap and mismatch count of 2, minimum neighborhood phred quality of 25, and minimum central quality of 30. Minimum coverage of called regions was set at 20, and minimum variant frequency was set to 5%. Only non-synonymous mutations were further pursued, whereas splice-site mutations were determined using the predict splice-site effect module. Average coverage of targeted regions was performed using the coverage analysis module and including only the targeted exons and not the flanking regions.

Analysis of histological sections

All stainings were performed at the central pathology review site, the Institute of Pathology at the University Hospital of Basel. Conventional hematoxylin and eosin staining was performed on deparaffinized sections of the formalin-fixed, paraffin-embedded and ethylene-diamine-tetra-acetate decalcified bone marrow biopsies of the study patients. For assessment of reticulin fibrosis, Gömöri staining on an automated stainer (Benchmark Special Stainer, Ventana) was utilized, applying the Reticulum II staining kit. Argyrophilic reticulin fibrosis was scored according to the European consensus guidelines,[3] which are now incorporated into the revised 2016 WHO classification.[4,5] Collagen fibrosis was assessed on Masson's Trichrome stained sections: the deparaffinized sections were incubated in Weigert's iron-hematoxylin, rinsed in water and differentiated in 0.5% HCl-ethanol solution, then blued in water and incubated in 50% acidic fuchsine Ponceau solution and 1% phosphomolybdic acid solution, stained with aniline blue, rinsed in water and 1% acidic acid and cover slipped. Collagen deposition was assessed according to recent guidelines.[6] Immunohistochemistry with the monoclonal antibody 10C2 from AbD Serotec (OBT1610) against nestin at a dilution of 1:50 and with the QBEnd/10 from Ventana (790-2927) against CD34 (ready to use kit) was

performed on an automated immunostainer (Benchmark XT, Ventana), as previously described.[7] The number of nestin+ perivascular niches (either single cells or clusters of up to 3 cells) was quantified on an average area of 7.2 mm^2 for each case and then referred to 1 mm^2. The reproducibility of the fibrosis scoring and nestin+ niches' quantification was tested on digital images taken from 4 case-pairs, which were scored in a blinded fashion by the same scoring pathologist after an interval of 2 months. CD34+ blasts were assessed as percentage counting 500 cells in the hotspot.

Statistical methods

A single-arm single-stage design was applied to test the null hypothesis that the proportion of patients with a reduction of at least 50% in *JAK2*-V617F allele burden at 24 weeks would be $\leq 30\%$ versus the alternative hypothesis that this proportion would be $\geq 50\%$. With $\alpha=0.05$ and 80% power, 39 evaluable patients were required. According to the design, the null hypothesis could be rejected, if at least 17 among the 39 patients had a reduction of at least 50% at 24 weeks. Continuous data were summarized using median, first and third quartiles. Categorical data were summarized using frequency counts and percentages. Differences were tested with the Wilcoxon rank-sum test, Fisher's exact test or McNemar's Test. For proportions, exact 95% confidence intervals (90% for the primary endpoint) were calculated. For time-to-event endpoints, the types and numbers of events were summarized descriptively, and the median value and the corresponding 95% confidence interval were calculated using the Kaplan-Meier method. Spearman's rank order correlation was used for calculation of correlation coefficients. All analyses were performed using SAS 9.4 (SAS Institute, Cary NC) and R 3.2.5.

REFERENCES

1. Barosi G, Mesa R, Finazzi G, et al. Revised response criteria for polycythemia vera and essential thrombocythemia: an ELN and IWG-MRT consensus project. Blood. 2013;121(23):4778-4781.
2. Tefferi A, Cervantes F, Mesa R, et al. Revised response criteria for myelofibrosis: International Working Group-Myeloproliferative Neoplasms Research and Treatment (IWG-MRT) and European LeukemiaNet (ELN) consensus report. Blood. 2013;122(8):1395-1398.
3. Thiele J, Kvasnicka HM, Facchetti F, Franco V, van der Walt J, Orazi A. European consensus on grading bone marrow fibrosis and assessment of cellularity. Haematologica. 2005;90(8):1128-1132.
4. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127(20):2391-2405.
5. Thiele J, Kvasnicka HM, Orazi A, et al. Myeloprolifierative Neoplasms. In: Swerdlow SH, Campo E, Harris NL, et al., eds. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon: International Agency for Research on Cancer (IARC); 2017:585.
6. Kvasnicka HM, Beham-Schmid C, Bob R, et al. Problems and pitfalls in grading of bone marrow fibrosis, collagen deposition and osteosclerosis - a consensus-based study. Histopathology. 2016;68(6):905-915.
7. Arranz L, Sanchez-Aguilera A, Martin-Perez D, et al. Neuropathy of haematopoietic stem cell niche is essential for myeloproliferative neoplasms. Nature. 2014;512(7512):78-81.

LEGEND FOR THE SUPPLEMENTAL DATA FILE

UPN = unique patient number

W0 = week 0

W12 = week 12

W24 = week 24

PV = polycythemia vera

ET = essential thrombocythemia

PMF = primary myelofibrosis

Post-PV MF = post polycythemia vera myelofibrosis

Post-ET MF = post essential thrombocythemia myelofibrosis