INTRODUCTION

Myeloproliferative neoplasms (MPN) are divided in different categories, mostly based on their clinical presentation.\(^1\) Besides BCR-ABL1-positive chronic myeloid leukemia (CML), essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF) represent the most frequent MPN subtypes. The three MPN sub-entities share the JAK2 driver gene mutation whereas MPL and CALR driver gene activations are restricted to ET and PMF.

Median survival is favorable in ET with approximately 20 years, whereas median life expectancy in PV and PMF is shorter (14 and 6 years, respectively).\(^2\) Furthermore, ET patients show a leukemic...
transformation only in 2%-5% of cases, and myelofibrosis (MF) occurs in 1.5% of patients within 10 years after diagnosis. Within 10-15 years after initial diagnosis, 20%-30% of PV patients develop MF and 10%-20% undergo progress to blastic phase of disease. In PMF, blastic transformation occurs in approximately 10%-20% of patients.

Several prognostic models were constructed over the last years, mostly based on clinical defined variables. Recently, a genetically inspired prognostic scoring system (GIPSS) for PMF was developed, which is reported to be superior in comparison with the clinical based scoring system. In the GIPSS model, CALR type 1/type 1-like mutations are a favorable genetic marker, whereas ASXL1, SRSF2, and U2AF1 p.Q157 mutations are associated with inferior survival. A similar prognostic model for ET and PV was developed by the same international consortium.

The aim of this study was to detect retrospectively genetic aberrations in patients at presentation with later blastic progression during course of disease. By comparison with a non-progressive cohort with a similar follow-up period, we expected to sort out genetic alterations which convey an increased risk for blastic progression in MPN patients as could already be demonstrated for the development of fibrosis in prefibrotic PMF.

2 | MATERIAL AND METHODS

2.1 | Patient samples

Decalcified formalin-fixed, paraffin-embedded bone marrow trephines were selected retrospectively from the archive of the Institute of Pathology. All selected cases underwent histopathological routine diagnostic procedures in our institute. For the blastic progression cohort (n = 38), cases were selected with at least two available bone marrow biopsies and a minimum of 1-year follow-up time. 16 cases were diagnosed with PMF, 11 cases with PV, and further 11 cases with MPN unclassified. The first bone marrow biopsy at the time of the initial diagnosis had to be myelofibrosis grade 0 (MF0) and no excess of blasts. The latest available biopsy showed excess of blasts-type 2 (10%-19%) or a transformation of the MPN disease into a secondary acute myeloid leukemia (2nd AML, blasts ≥ 20%) according to the WHO classification, with or without myelofibrosis.

The cohort without blast excess progression (n = 63) consists of MPN cases with at least two available bone marrow biopsies and a minimum of 1-year follow-up time. First bone marrow biopsy at the time of the initial diagnosis had to be MF grade 0 and no observable blast excess. In addition, also the last available biopsy had to show no MF or blast excess. The cohort without progression consisted of two already published sample sets with PMF (n = 27) and PV (n = 36) cases. No other concomitant neoplasms were present in any selected case. In the cohort without progression, the reasons to perform follow-up bone marrow biopsies were heterogeneous, for instance anemia or thrombosis. Fibrosis was determined by Gomori staining as described. Immunohistochemical staining was done on every case and encompassed CD3, CD20, CD34, CD42, and CD117.

The study design is following the guidelines of the Hannover Medical School ethics committee.

2.2 | Nucleic acid extraction and sequencing

Extraction of DNA and sequencing analysis were performed as described previously on an Ion S5 instrument (LifeTechnologies). A local Ion Reporter server (Version 5.12.1) was used to analyze the .bam files. Minimum variant coverage was 500, minimum allelic frequency was 3%, and the allowed strand bias was 0.9 or greater. All .tsv variant lists were checked manually to discriminate single-nucleotide polymorphisms from the somatic variants and pathogenic mutations. Targeted sequencing of n = 25 genes was performed using a NGS amplicon based panel consists of 23 genes and pyrosequencing for MPL Codon p.W515 and ETNK1 Codon p.N244 as described.

2.3 | Bioinformatic classification of variants and biostatistics

Sequencing data evaluation and variant annotation were performed with the ANNOVAR software and database tools (http://annovar.openbioinformatics.org/en/latest/). Variants with unknown significance had to be predicted as deleterious/disease causing in all of the following in silico prediction tools in order to be considered as pathogenic: MutationTaster, SIFT, PolyPhen, MutationAssessor and a CADD_pred value above 20. Statistical analysis was performed with GraphPad Prism version 5.00, and two-sided Fisher’s exact test and Mann-Whitney U test were used (results are considered to be
statistically significant when $P < .05$). Kaplan-Meier survival curves (two groups) were calculated with a Log-rank (Mantel–Cox) Test (results are considered to be statistically significant when $P < .05$).

## 3 RESULTS

### 3.1 Gene mutations associated with blastic progression

Clinical parameters of both cohorts are listed in Table 1 (details for all patients in the blastic progression cohort are listed in Table S1). Gender, age at diagnosis, and mean follow-up times are comparable between both cohorts. Mean number of mutations at diagnosis were significantly higher in patients with later blastic progression than in patients with stable disease during follow-up (2.08 vs. 1.38 mutations, $P = .0002$). Individual gene mutations per patient at time of MPN diagnosis in the blastic progression cohort are shown in Figure 1 (see Table S1 for details). The majority of cases harbor a JAK2 mutation as MPN driver mutation (28/38, 73.7%). Only one case each shows a CALR or MPL mutation. Besides the typical MPN driver gene mutations, a spectrum of further mutated genes relevant in hematologic malignancies could be detected. Seven cases harbor SRSF2 (all affecting exon 1, p.P95H/R/L) and five cases U2AF1 hotspot mutations (all affecting exon 6, p.R156H and p.Q157R/P, see Table S1), mutually exclusive. Furthermore, IDH1/2 hotspot mutations could be detected in five patients at time of diagnosis. However, four patients with IDH1/2 mutations show either a SRSF2 or U2AF1 hotspot mutation in combination. ASXL1, DNMT3A, and TET2 mutations could be detected in five, two, and twelve patients, respectively (Figure 1). There are no observable differences of gene mutations in patients with excess of blasts-type 2 (10%-19%) and

### Table 1 Clinical parameters and sequencing results of the MPN blastic progression cohort and the MPN cohort without blast excess

|                      | MPN with blast excess | MPN without blast excess | $P$ value |
|----------------------|-----------------------|--------------------------|-----------|
| n                    | 38                    | 63                       |           |
| Gender (female)      | 47.4%                 | 55.5%                    |           |
| Age (at diagnosis)   | 66.1 y                | 62.6 y                   |           |
| Follow-up total      | 201.5 y               | 362.5 y                  |           |
| Range                | 1.0-16.0 y            | 1.0-14.0 y               |           |
| Mean follow-up       | 5.3 y                 | 5.8 y                    |           |
| Hemoglobin (g/dL, median), first|last biopsy | 14.4|9.5 16.1|12.9 |
| Leukocytes ($\times10^6xL^{-1}$), first|last biopsy | 20.2|19.6 10.2|16.5 |
| Thrombocytes ($\times10^9xL^{-1}$), first|last biopsy | 552|196 715|560 |
| Spleen size (cm), first|last biopsy | 15.0|15.0 13.3|14.4 |
| Mean number of mutations (range) | 2.08 (0-5) 1.38 (0-3)***($P = .0002$) | MWU |
| Standard error of mean (SEM) | 0.174 0.094 |           |
| Cases with clonal evolution during follow-up | 18/38 3/63 ***($P < .0001$) | FES |
| Gene mutations (at diagnosis) | JAK2 28/38 51/63 ns | FES |
|                      | CALR type 1 0/38 3/63 ns | FES |
|                      | CALR type 2 1/38 3/63 ns | FES |
|                      | MPL 1/38 1/63 ns | FES |
|                      | JAK2/CALR/MPL wt 8/38 5/63 ns ($P = .0703$) | FES |
|                      | ASXL1 5/38 3/63 ns | FES |
|                      | DNMT3A 2/38 7/63 ns | FES |
|                      | TET2 12/38 14/63 ns | FES |
|                      | SRSF2 7/38 0/63 ***($P = .0007$) | FES |
|                      | U2AF1 5/38 0/63 **($P = .0063$) | FES |
|                      | IDH1/2 5/38 0/63 **($P = .0063$) | FES |
|                      | EZH2 3/38 0/63 ns ($P = .0506$) | FES |

Abbreviations: FES, Fisher’s exact test; MWU, Mann-Whitney-U test; ** statistically significant $<.01$; *** statistically significant $<.001$. 
In Figure 2, gene mutations at time of diagnosis from patients with stable disease in follow-up are shown. The cohort is divided into PMF (n = 27) and PV patients (n = 36). JAK2 is the mostly detected MPN driver mutation (51/63, 80.9%). CALR mutations are present in six PMF patients (three type 1 and type 2 mutations each). One patient harbors a MPL hotspot mutation. MPN cases without typical MPN driver mutation (so-called triple-negative MPN) are present in 5/63 (7.9%) patients of the stable disease cohort and in 8/38 (21.1%) patients of the blastic progression cohort (P = .0703, ns, Table 1). ASXL1, DNMT3A, and TET2 mutations could be detected.

**FIGURE 1** Detected mutations at time of initial diagnosis in the patient cohort with blastic progression during follow-up (n = 38). MF: myelofibrosis grade, EB: grade of blasts excess in the bone marrow. EB2: excess of blasts-type 2 (10%-19%). EB3: transformed MPN disease which fulfills the criteria of a secondary acute myeloid leukemia (2nd AML, blasts ≥ 20%).
FIGURE 2  Detected mutations at time of initial diagnosis in the patient cohort with stable disease (SD) during follow-up. In total, this cohort consists of 63 patients, divided into PMF \( (n = 27, \text{upper cases}) \) and PV \( (n = 36, \text{lower cases}) \).
in three, seven, and thirteen patients with stable disease, respectively (Figure 2).

Comparing gene mutations in both cohorts, SRSF2, U2AF1, and IDH1/2 hotspot mutations are significantly correlated with blastic progression in follow-up (P = 0.0007 for SRSF2, P = 0.0063 for U2AF1 and IDH1/2, Table 1). Genes which are frequently mutated in ARCH/CHIP (ASXL1, DNMT3A, and TET2) are not correlated with blastic progression during follow-up (Table 1).

### 3.2 Rapid blastic progression of SRSF2 and U2AF1 mutated MPN

Beside the significant correlation of SRSF2 and U2AF1 mutations with later blastic progression, also time to progression itself is shortened. Figure 3 shows the follow-up time to blast progression type 2 (10%-19%) or transformation into a 2nd AML (blasts ≥ 20%). Cases with SRSF2 and U2AF1 mutations (n = 12) had a mean time to progress of 1.5 years, whereas cases without these mutations show a mean time of 6.8 years until blastic progression is manifest (P = 0.0031). IDH1/2 mutations are not considered separately in this analysis because of their frequent co-occurrence (4/5, 80%) with SRSF2 and U2AF1 mutations.

### 3.3 Clonal evolution in MPN with blastic progression

In Figure 4, clonal evolution (de novo mutations and eradication of mutated clones in follow-up) of patients with blastic progression are shown. Most frequently, acquired TP53 mutations could be detected in progressive samples (9/38, 23.7%). Furthermore, three patients developed RUNX1 mutations. All other genes covered show acquired mutations in follow-up only occasionally. No cases with an acquired SRSF2 mutation in blast phase could be detected, whereas in two patients we could detect a de novo SF3B1 and U2AF1 mutation, respectively.

**FIGURE 3** Kaplan-Meier plot showing the time to manifest blast progression of cases with blastic progress during follow-up. The cohort was divided into cases with (n = 12) and without (n = 26) SRSF2 and U2AF1 mutation for the purposes of this analysis.

### 4 DISCUSSION

Recently, we could show that mutations rarely found in age-related clonal hematopoiesis (SRSF2, SF3B1, U2AF1, IDH1/2, and EZH2) are associated with later fibrotic progression in PV and PMF patients. These mutations are already present at diagnosis of MPN disease and therefore useful as negative prognostic markers. Furthermore, patients with these mutations show a rapid fibrotic progress in comparison with those patients with frequent ARCH/CHIP mutations (ASXL1, DNMT3A, and TET2) or only MPN driver mutations (JAK2, CALR, MPL) detectable at diagnosis. In the present study, we focused on blastic progression in MPN cases and could show associations between genetic alterations at time of diagnosis and disease progress in follow-up similar to fibrotic progression.

Splice factor mutations in SRSF2 and U2AF1 are already present at time of diagnosis in patients who undergo a later blastic progression. For SRSF2 mutations, the association with increased leukemic transformation and poor overall survival in PMF is described. U2AF1 mutations are associated with inferior overall survival in PMF and have a negative prognostic impact in patients with ET.

MPN cases without detectable driver mutation in JAK2, MPL, or CALR, so-called triple-negative, show a trend to later blastic progression (21.1% vs. 7.9%, P = 0.0703, ns, Table 1). This confirms the overall worse prognosis of triple-negative MPN. Additionally, we could detect only one CALR mutated patient in the blastic progression cohort (n = 38), compared with six patients harbor CALR mutations in the PMF sub-cohort of patients without progress (n = 27). Despite of the limited number of cases, this is well in line with a positive prognostic effect in CALR mutated MPN cases. However, prognostic effect of CALR mutations in general and Type 1/1- like and 2/2- like mutations in particular are still controversial.

Interestingly, three cases (ID 1, 7, and 10) harbor EZH2 mutations at presentation (one case with concomitant U2AF1 and IDH2 mutations) and all showed a blastic progression within 1 year after initial diagnosis (Figure 1). No EZH2 mutation could be detected in
the stable disease cohort (Figure 2). The association with an EZH2 mutation at time of diagnosis and later blastic progress is of borderline significance in our study ($P = .0506$, two-sided Fisher’s exact test, Table 1). Mutated EZH2 is known as negative prognostic marker for leukemic transformation in MPN disease.\textsuperscript{25,26} Taken together, the presence of EZH2 mutations only in progressive cases and the rapid blastic progression within 1 year are strong arguments for a negative prognostic effect, despite the missing significance in our cohorts, which are quite limited in number.

Controversially, ASXL1 mutations alone are not associated with later blastic progression in our study. In chronic myelomonocytic leukemia, ASXL1 mutations (and DNMT3A mutations) have a negative impact on overall survival.\textsuperscript{27} Vallapureddy et al identified in 1306 PMF patients IDH1, SRSF2, and ASXL1 mutations being associated with leukemic transformation. However, ASXL1 had the lowest hazard ratio (HR) compared with SRSF2 and IDH1 (HR 2.0, HR 3.0, and HR 4.3) in their cohort.\textsuperscript{28} A large-scale cohort analysis of patients with clonal hematopoiesis show that presence of
ASXL1-mutant clones is significantly associated with current and past smoking.29

Recently, Luque Paz and colleagues30 presented a data set comparable to our study with n = 49 leukemic transformed PV and ET cases (DNA samples from time of diagnosis were available for 21 patients) and n = 80 patients in the control group (at least 8 years of follow-up without transformation). In this study, the authors defined three molecular risk groups with distinct time to leukemic transformation. Gene mutations with high frequencies were found in TP53 (12/49, 24.5%), splicing genes (11/49, 22.5%), RUNX1 (10/49, 20.4%), and IDH1/2 (8/49, 16.3%) at the time of leukemic transformation. These results are highly comparable with our findings at time of leukemic transformation (TP53 26.3%, splicing genes 36.8%, RUNX1 13.2%, and IDH1/2 15.8%, Figure 4). In the control group of Luque Paz et al,30 10 cases show TET2 mutations, TET2 was consequently excluded from further statistical risk stratification analysis. Furthermore, DNMT3A mutations were found in 4/80 cases in the control group. Considering this, the results of Luque Paz et al confirm our findings that TET2 and DNMT3A mutations at time of MPN diagnosis are not significantly associated with later leukemic transformation. ASXL1 mutations are present in 4/21 patient samples with later leukemic transformation at time of diagnosis and in 1/80 cases in the control group,30 considering a higher risk of ASXL1 mutated patients for later leukemic transformation. Nevertheless, ASXL1 mutations were found to be equally distributed in all three molecular risk groups.30

Clonal evolution (de novo mutation or eradication of a mutated clone during follow-up) is a frequent event in MPN patients with later blastic progression in our study (18/38, 47.4% vs. 3/63, 4.7%, P < .0001, Table 1). Especially de novo TP53 and RUNX1 mutations are associated with blasts excess (Figure 4). However, the low frequency of TP53 and RUNX1 mutations at time of diagnosis limit their potential as prognostic markers. The overall mutation rate in MPN disease is rather low,31 but patients with fibrotic or blastic progression in follow-up show a dynamic picture of acquiring and eradicated mutated clones (12 and Figure 4).

We could found a combination of splice factor gene mutations (SRSF2 and U2AF1) with epigenetic modifier gene mutations (IDH1/2) in patients with a rapid leukemic transformation. The association of SRSF2 and IDH1/2 gene mutations is known especially for PMF.32 Jak2/Idh-mutated mice do not show overt leukemia,32,34 suggesting that additional gene mutations like SRSF2 and U2AF1 are required for a leukemic transformation. Furthermore, Srsf2 p.P95H mutant in mice lead to mis-splicing and nonsense-mediated decay of Ezh2.32,35 EZH2 and SRSF2 genes are exclusively mutated in our leukemic transformation cohort, suspecting a redundancy in the disease mechanism.

A clear limitation for comparative studies of MPN cohorts is the lack of regularly taken bone marrow biopsies from long-term chronic phase patients without disease progress. Time to blastic progression could be decades and the mean follow-up time of patients in our cohort without blastic progression is 5.8 years in our study. Therefore, it could not be excluded that some patients of the cohort without blastic progression will undergo a progression in the future, may be with consequences for the presented results.

In conclusion, we could show that mutations in SRSF2, U2AF1, IDH1/2, and may be EZH2 can serve as negative prognostic markers for rapid blastic progression in newly diagnosed MPN. Therefore, follow-up bone marrow biopsies of these high risk patients in short-time intervals are highly recommended.

ACKNOWLEDGEMENTS

Open access funding enabled and organized by ProjektDEAL.

CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

AUTHOR CONTRIBUTIONS

Contribution: SB, UL, and HK planned the study, interpreted data, and wrote the paper; SB, JV, and ES performed experiments and analyzed the data; GB JS, and HK contributed the histopathological diagnosis of the patient samples.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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**How to cite this article:** Bartels S, Vogtmann J, Schipper E, et al. Combination of myeloproliferative neoplasm driver gene activation with mutations of splice factor or epigenetic modifier genes increases risk of rapid blastic progression. *Eur J Haematol*. 2021;106:520–528. [https://doi.org/10.1111/ ejh.13579](https://doi.org/10.1111/ ejh.13579)