The poor outcome in high molecular risk, hydroxycarbamide resistant/intolerant essential thrombocythemia is not ameliorated by ruxolitinib treatment: The MAJIC-ET Cohort

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Essential Thrombocythemia (ET) is a myeloproliferative neoplasm (MPN) defined by thrombocytosis, increased risk of vascular thrombosis,[1,2] hemorrhage[3] and progression to myelofibrosis (MF)[4,5] and acute myeloid leukemia (AML).[4,5] Patients are risk-stratified to identify those who might benefit from cytoreduction to reduce the risk of vascular complications.[6] Resistance/intolerance to hydroxycarbamide (HC-RES/INT), a first-line cytoreductive treatment, develops in 20% of high-risk patients[7] with increased risk of disease progression and reduced survival.[8] New approaches are needed to predict disease transformation risk in these patients, together with development of therapies that reduce this risk.

Following the discovery of the Janus Kinase 2 *(JAK2*) mutation (*JAK2*V617F), present in ~50% of ET,[9] the first approved JAK1/JAK2 inhibitor, Ruxolitinib (RUX), is now widely used for treatment of myelofibrosis[10] and polycythemia vera.[11] The MAJIC trial explored the role of RUX in HC-RES/INT ET, randomizing patients 1:1 to RUX or best available therapy (BAT), demonstrating similar rates of 1-year complete hematological response (CHR).[12] Mutational status was not comprehensively reported in this paper. This is important as ET patients (29-72%)[13,14] carry mutations in non-MPN driver genes (NDM). Inferior prognosis is associated with specific mutations at diagnosis.[14] The impact of NDM in HC-RES/INT ET is unknown, as is the effect of RUX on disease course in molecularly defined subgroups. We therefore evaluated mutational status of MAJIC-ET patients and correlated this with clinical outcomes.

Next generation sequencing (NGS) was performed at baseline (n=110) and serially if a later sample was available (see Supplemental Methods for NGS and statistical analysis methodology). Median follow-up was 55 months (95% confidence interval [CI], 49.9–60.4). *JAK2*, *CALR* and *MPL* mutations were present in 49.1%, 30% & 4.5% of patients, respectively and 16.4% of patients were “triple-negative” (TN). Baseline NDM were present in 30% (n=33) of patients with >1 present in 10% (Figure 1A), most frequently *TET2* (n=12), *TP53* (n=7) and *SF3B1* (n=7) genes (Figure 1B; Supplemental Table 1). Driver mutation variant allele frequency (VAF) was higher than NDM VAF in 66.67%, 87.5% and 20% of *JAK2*, *CALR* and *MPL*-mutated patients respectively (Figure 1C).[15] Patients with NDM tended to be older with lower hemoglobin levels (Figure 1D, Supplemental Table 2). *TP53* mutations trended towards a higher frequency in TN (17.6%) than in *JAK2/CALR/MPL*-mutated patients (4.3%), p=0.073. In the primary analysis, driver mutation status did not correlate with CHR[12]. Since platelet count reduction is a key therapeutic goal, we performed a post-hoc analysis defining platelet response as <400 x 10^9/l at 1-year. RUX-treated *JAK2*V617F-mutated patients had significantly more platelet responses than *JAK2*V617F wild-type (WT) patients, a difference not seen for BAT-treated patients (Figure 1E). RUX discontinuation was most often due to treatment failure and this correlated with non *JAK2*V617F-mutated status (OR 6.1 [95% CI 1.43–26.6], p=0.015). NDMs did not influence hematological/symptom responses (Supplemental Table 3).

Transformation events occurred in 12.7% (Supplemental Table 3). *TP53*-mutated patients had inferior 4-year transformation-free survival (TFS) of 42.9% (95% CI 9.8–73.4%) versus 79.8% (95% CI 69.7–86.8%) for WT patients, p=0.011 (Figure 2A). Splicing factor (SF) mutations conferred a poorer 4-year TFS of 40% (95% CI 12.3–67%) versus 81.5% for WT patients (95% CI 71.4–88.3%; p=0.00039, Figure 2B); predominantly attributable to mutated-*SF3B1* (p=0.004). High molecular risk (HMR) mutations in this cohort (defined by SF and

TP53 mutations) conferred a poorer TFS (p<0.0001, Figure 2C) which was not ameliorated by RUX (Figure 2D). HMR mutations retained their negative impact on multivariable analysis (Figure 2E). Driver mutation VAF ≥50% and male gender independently conferred a poorer TFS, findings reported by other groups.[16,17] Mutated-*TET2* did not correlate with clinical outcomes, comparable to previous findings.[14]

Thrombotic (19.1%, n=21/110) were not influenced by mutational status overall. This is in contrast to previous studies reporting a greater thrombotic risk in *JAK2*V617F-mutated patients.[4] Whilst a possible explanation is that this association is not seen in HC-RES/INT patients, the number of events in our study is small and should therefore be interpreted with caution. Hemorrhagic events (9.1%, n=10/110) were specifically associated with SF mutations, p=0.007 (Supplemental Table 3). Grade 3/4 hematological toxicities were not associated with mutational status. Overall survival at 4-years of 91.5% (95% CI 80.2-96.4%) in BAT and 83% (95% CI 70.4-90.5%) in RUX arms (p=0.22) was not influenced by mutational status.

1-year driver mutation molecular responses (MR) were rare (n=3), occurring exclusively in the RUX arm; a complete MR (CMR) in 2 patients *(JAK2*V617F-mutated and *CALR*-mutated) and one *CALR*-mutated partial MR (PMR). Longitudinal driver mutation analysis was performed in 54% (n=50/93); median analysis time 48 (24–60) months with no significant change in VAF at any time point (Supplemental Figure 1A & B). 1-year MR was lost in 2 patients (Supplemental Figure 1C & D). Longitudinal NDM analysis was possible in 52% (n=57/110); median analysis time 40 (6-60) months. New NDM, defined by identification at VAF ≥5%, were detection in 19.3% (n=11/57) at a similar frequency across treatment arms (Supplemental Table 4) and no significant correlations were detected with baseline NDM or clinical/survival outcomes. However, a median follow-up time of 10.7 months (95% CI 9.05–12.4) after later NDM analysis is not sufficient time for survival analysis. These data highlight the clinical utility of serial molecular analysis in HC-RES/INT ET.

In this analysis, we identify NDM at baseline in 30% of patients, a higher frequency than most previous analyses, which may relate to this high-risk nature of this cohort.[13,14,16,18] *TP53* and *SF3B1* mutations were observed each at 6.4%, higher than previously reported in ET (~2 and 2-5% respectively).[13,14,16,19] The frequent detection of *TP53* mutations in TN patients was unexpected but the numbers are too few (n=3) to draw firm conclusions. Disease transformation was specifically associated with SF (most commonly *SF3B1*) and *TP53* mutations, determining a HMR for this cohort. However, this requires independent validation

in larger cohorts before being applied in clinical practice. *TP53* mutations in MPNs have been associated with AML transformation[14,16] but have not been reported to increase myelofibrotic transformation in ET.[14,16] Myelofibrotic transformation has been reported in association with SF mutations in ET, most often mutated-*SF3B1,*[14,20] but a recent large MPN study, identified *SRSF2, ZRSR2* and *U2AF1* but not *SF3B1*[16] as myelofibrotic transformation predictors in ET. This contrasts with myelodysplastic syndromes where *SF3B1* mutations confer better survival[21-23] with lower risk of disease progression[21] suggesting disease context and co-mutations (primarily *JAK2*V617F here) are relevant.

Importantly, disease transformation in patients with HMR-NDM was not mitigated by RUX which is noteworthy as there has been interest in the possibility that early intervention with JAK2 inhibition might attenuate disease progression. We observed a novel association between SF mutations and hemorrhagic events; this finding needs independent corroboration due to low event rate. We also found that *JAK2*V617F-mutated status correlated with improved platelet responses to RUX, and notably, more non-*JAK2*V617F mutated patients stopped RUX raising the possibility that *JAK2*V617F-mutated ET patients might selectively benefit from RUX.

In summary, we report for the first time, comprehensive mutational analysis of HC-RES/INT ET within the context of a prospective randomized clinical trial. We found a particularly high prevalence of *TP53* and splicing factor mutations, which was strongly predictive of subsequent disease transformation, and was not mitigated by RUX. This highlights the clinical/prognostic utility of serial mutation screening in HC RES/INT ET to allow identification of patients at risk of disease transformation.

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Authorship Contributions: J.M.O'S. analyzed experiments, performed the statistical analysis and wrote the manuscript. A.H. oversaw the myeloid gene panel analysis and contributed to writing of the manuscript. R.B. and A.P. were involved in the statistical analysis with senior oversight from C.Y. S.A. and S.F. contributed to data collection. H.D., K.H. and P.W. processed samples, performed experiments and analyzed data. N.C.P.C. analyzed experiments. M.F.McM. contributed to data analysis. C.N.H. conceived and supervised the project and contributed to writing the manuscript. A.J.M. conceived and supervised the project, designed and analyzed experiments and wrote the manuscript. All authors read and approved the submitted manuscript.

Conflict-of-interest disclosure: A.J.M. has participated in advisory boards for Novartis, CTI, and Baxaltra; received honoraria from Novartis, Gilead, Shire, and Baxaltra; and also received research funding and travel, accommodation, and expenses from Novartis. A.H. has participated in advisory boards for Novartis; received honoraria from Gilead, Pfizer and Roche. N.C. received honoraria for Novartis, Pfizer and Incyte. C.Y. received honoraria from Celgene.

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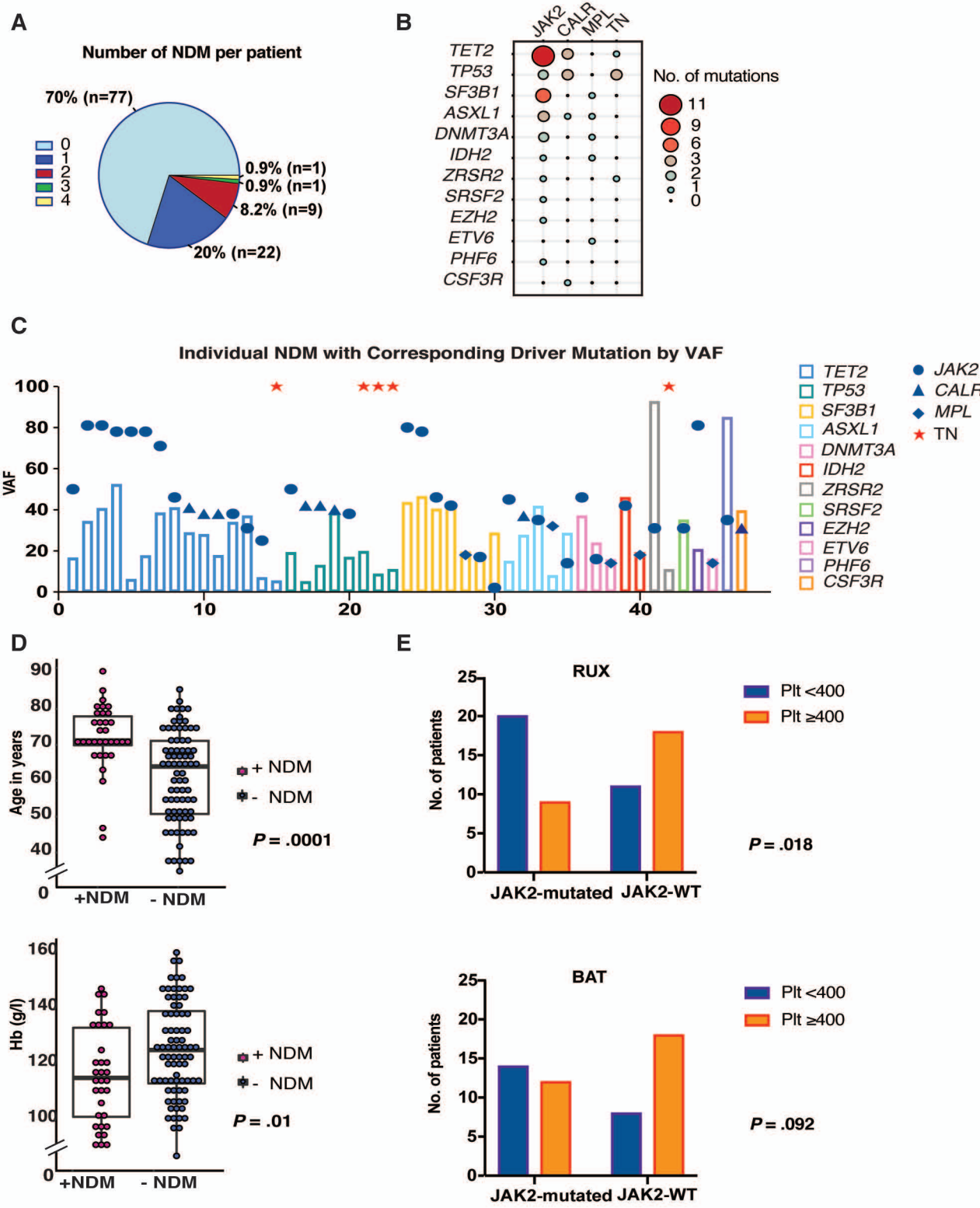
Figure Legends

Figure 1. Baseline mutational analysis and correlation with clinical characteristics and treatment response. (A) Pie chart showing number of NDM per patient. (B) Balloon plot showing association of driver mutations with NDM with size and colour of bubble corresponding to frequency of association; NDM were more often associated with *JAK2*V617F mutations. (C) Column and dot plot showing variant allele frequencies (VAF) of each NDM (column) with corresponding driver mutation (blue dot). Red star indicating TN patient; driver mutation VAF was higher in 66.67%, 87.5% and 20% of *JAK2*, *CALR* and *MPL*-mutated patients suggesting driver mutation acquisition first in these, although with the caveat that order of mutation acquisition can only be definitively assigned using single-cell methodologies. (D) Dot and box plots of median age at trial entry in patients with NDM compared to patients without NDM; 71 vs. 64 years, p=0.0001 (upper plot) and hemoglobin (Hb) level (mean Hb 115g/l) lower in patients with NDM compared to patients without NDM (mean Hb 125g/l), p=0.01 (lower plot). Dots represent each individual patient and each horizontal line and box represent the median for age/mean for Hb and interquartile ranges respectively using Mann-Whitney U test to compare median ages (non-normal distribution) and Student's t-test to compare Hb means (normal distribution). (E) Post hoc analysis of 1-year platelet count responses; significantly more patients on RUX who were *JAK2*-mutated achieved plt ≤400 than non *JAK2*-mutated patients (upper bar chart). This difference was not seen within the BAT arm (lower bar chart). BAT=best available therapy; *JAK2*=*JAK2*V617F;

NDM=non-MPN driver mutation; Plt ≤400=platelet count of ≤400 x 10^9/l; Plt >400=platelet count of > 400 x 10^9/l; RUX=ruxolitinib; TN=Triple negative.

Figure 2. Kaplan-Meier curves of transformation-free survival (TFS) stratified by mutational statuses with survival estimates, reported at 4-years. (A) *TP53* mutations were associated with inferior 4-year TFS; *TP53*-mutated (42.9% [95% CI 9.8 – 73.4%]) vs. *TP53*-wild type (WT) patients (79.8% [95% CI 69.7 – 86.8%]), p=0.011. (B) SF mutations conferred a poorer 4-year TFS; SF-mutated (40% [95% CI 12.3 – 67%]) vs. SF-WT (81.5% [95% CI 71.4 – 88.3%]), p=0.00039. (C) Comparing patients with HMR with LMR at 4-years; HMR 41.2% (95% CI 23.3-72.7%) vs. LMR 84.6% (95% CI 76.9 – 93.1%), p<0.0001. (D) Stratifying patients with high risk molecular (HMR) mutations in this study by treatment arm demonstrates no amelioration of negative impact of HMR mutation with RUX treatment; patients with HMR on RUX had TFS at 4-years of 36.4% (95% CI 26.2 – 46.6%) and on BAT 50% (29.1 – 67.7%) (p=0.505 between these arms) as compared to those without these mutations (i.e. low molecular risk, LMR) with TFS at 4-years of 84.7% (95% CI 71.6 – 92%) on RUX and of 90.6% (95% CI 78.5 – 96%) on BAT (p=0.101 between these arms). The log-rank test was used to compare survival estimates between groups. (E) Forest plot showing multivariable cox model of transformation-free survival (TFS). Covariates significant on univariate analysis were included; *TP53* mutations, SF mutations, treatment arm, *JAK2*V617F mutation status, disease duration at trial entry (TE), age and gender. HMR mutations independently retained negative impact on TFS with a hazard ratio (HR) of 4.21, p=0.006. Treatment arm, *JAK2*V617F status, disease duration at TE and age were not significant but notably male gender was associated with a poorer TFS, HR 4.5, p=0.006. Driver mutation allele ≥50% was independently associated with a poorer TFS, HR 4.11, p=0.016. Age and disease duration at TE were categorized as continuous variables. CI=confidence interval; HR=hazard ratio; HMR=high molecular risk risk (SF and *TP53* mutations); LMR=low molecular risk (without SF or TP53 mutations); *JAK2=JAK2*V617F; NDM=non-MPN (myeloproliferative neoplasm) driver mutation; SF=splicing factor mutation (*SF3B1, ZRSR2, SRSF2*); WT=wild type.

Figure 1.


A
Number of NDM per patient
70% (n=77)
0
1
2
3
4
0.9% (n=1)
0.9% (n=1)
8.2% (n=9)
20% (n=22)
B
JAK2
CALR
MPL
TN
TET2
TP53
SF3B1
ASXL1
DNMT3A
IDH2
ZRSR2
SRSF2
EZH2
ETV6
PHF6
CSF3R
No. of mutations
11
9
6
3
2
1
0
C
Individual NDM with Corresponding Driver Mutation by VAF
VAF
100
80
60
40
20
0
0
10
20
30
40
TET2
TP53
SF3B1
ASXL1
DNMT3A
IDH2
ZRSR2
SRSF2
EZH2
ETV6
PHF6
CSF3R
JAK2
CALR
MPL
TN
D
Age in years
90
80
70
60
50
40
0
+NDM
- NDM
+ NDM
- NDM
P = .0001
Hb (g/l)
160
140
120
100
0
+NDM
- NDM
+ NDM
- NDM
P = .01
E
RUX
No. of patients
25
20
15
10
5
0
JAK2-mutated
JAK2-WT
Plt <400
Plt ≥400
P = .018
BAT
No. of patients
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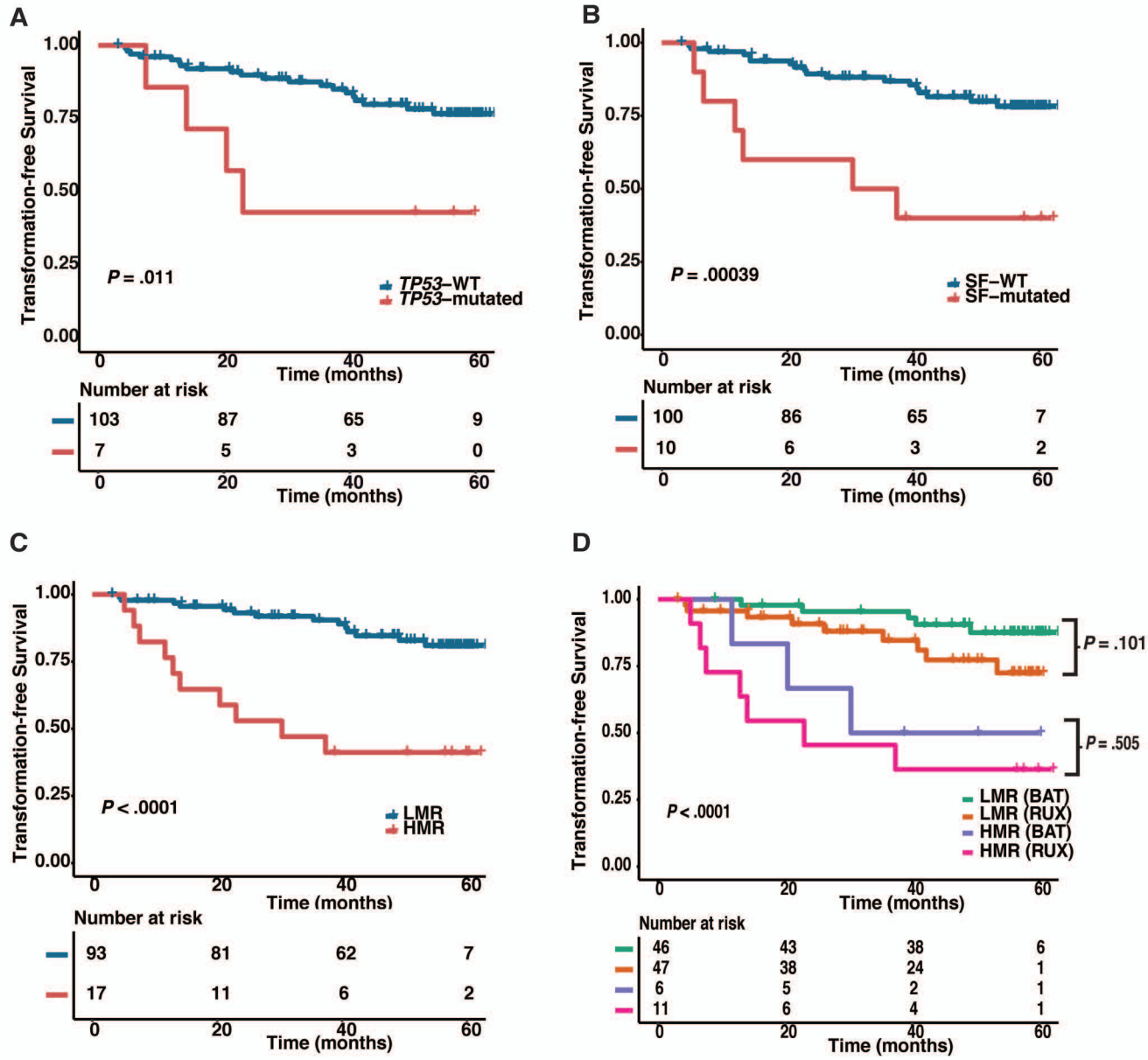
20
15
10
5
0
JAK2-mutated
JAK2-WT
Plt <400
Plt ≥400
P = .092

Figure 2.


A
Transformation-free Survival
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0.75
0.50
0.25
0.00
P = .011
TP53–WT
TP53–mutated
0
20
40
60
Time (months)
Number at risk
103 87 65 9
7 5 3 0
B
Transformation-free Survival
P = .00039
SF–WT
SF–mutated
Number at risk
100 86 65 7
10 6 3 2
C
Transformation-free Survival
P < .0001
LMR
HMR
Number at risk
93 81 62 7
17 11 6 2
D
Transformation-free Survival
P < .0001
P = .101
P = .505
LMR (BAT)
LMR (RUX)
HMR (BAT)
HMR (RUX)
Number at risk
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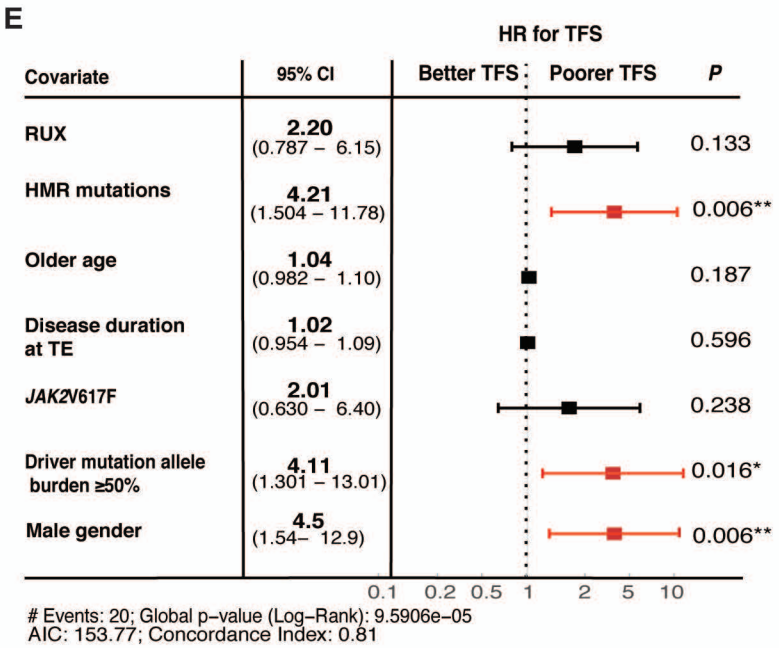
47 38 24 1
6 5 2 1
11 6 4 1


E
HR for TFS
Covariate
95% CI
Better TFS
Poorer TFS
P
RUX
2.20 (0.787 – 6.15)
0.133
HMR mutations
4.21 (1.504 – 11.78)
0.006**
Older age
1.04 (0.982 – 1.10)
0.187
Disease duration at TE
1.02 (0.954 – 1.09)
0.596
JAK2V617F
2.01 (0.630 – 6.40)
0.238
Driver mutation allele burden ≥50%
4.11 (1.301 – 13.01)
0.016*
Male gender
4.5 (1.54– 12.9)
0.006**
0.1 0.2 0.5 1 2 5 10
Events: 20; Global p–value (Log–Rank): 9.5906e–05
AIC: 153.77; Concordance Index: 0.81

Supplemental Tables and Figures

Supplemental Table 1. List of baseline NDM including genomic position, nucleotide change, protein consequence, mutation type and variant allele frequency (VAF).

ID	Gene	Genomic position	Nucleotide change	Protein consequence	Mutation type	VAF (%)
35	*TET2*	4:106157960	c.2861G>A	p.Trp954Xaa	SNV; nonsense	52.39
35	*TET2*	4:106164860	c.3729_3733delACT	p.Tyr1245GlyfsTer21	Frameshift del	6.18
35	*TET2*	4:106193999	c.4462delA	p.Asn1489MetfsXaa82	Frameshift del	17.74
54	*TET2*	4:106155920	c.822delC	p.Asn275IlefsXaa18	Frameshift del	38.55
55	*TET2*	4:106197236	c.5570_5571delCT	p.Pro1857ArgfsXaa17	Frameshift del	7.06
106	*TET2*	4:106155301	c.202G>T	p.Gly68Xaa	SNV; nonsense	37
108	*TET2*	4:106164939	c.3803+4A>T		Frameshift ins	28.86
128	*TET2*	4:106156540	c.1441C>T	p.Gln481Xaa	SNV; nonsense	41.35
131	*TET2*	4:106180796	c.3824G>T	p.Gly1275Val	SNV; missense	33.92
148	*TET2*	4:106162586	c.3500G>A	p.Arg1167Lys	SNV; missense	28.4
180	*TET2*	4:106196446	c.4781del	p.Pro1594LeufsXaa2	Frameshift del	16.61
184	*TET2*	4:106156365	c.1270del	p.Ser424AlafsXaa3	Frameshift del	5.47
213	*TET2*	4:106180829	c.3857C>T	p.Ser1286Phe	SNV; missense	34.35
213	*TET2*	4:106180854	c.3882C>G	p.Tyr1294TXaa	SNV; nonsense	40.71
215	*TET2*	4:106156812	c.1716dup	p.His573SerfsTer10	Frameshift ins	17.79
35	*SF3B1*	2:198266834	c.2098A>G	p.Lys700Glu	SNV; missense	46.58
73	*SF3B1*	2:198267483	c.1874G>T	p.Arg625Leu	SNV; missense	28.77
87	*SF3B1*	2:198267359	c.1998G>C	p.Lys666Asn	SNV; missense	43.62
103	*SF3B1*	2:198267359	c.1998G>C	p.Lys666Asn	SNV; missense	41.33
151	*SF3B1*	2:198267359	c.1998G>C	p.Lys666Asn	SNV; missense	40.38
193	*SF3B1*	2:198267359	c.1998G>C	p.Lys666Asn	SNV; missense	19.56
123	*SF3B1*	2:198267360	c.1997A>G	p.Lys666Arg	SNV; missense	17.2
27	*TP53*	17:7578406	c.524G>A	p.Arg175His	SNV; missense	19.8
38	*TP53*	17:7577545	c.736A>G	p.Met246Val	SNV; missense	38.46
118	*TP53*	17:7577578	c.703A>G	p.Asn235Asp	SNV; missense	5.09
118	*TP53*	17:7577568	c.713G>A	p.Cys238Tyr	SNV; missense	13.01
131	*TP53*	17:7577556	c.725G>A	p.Cys242Tyr	SNV; missense	16.98
158	*TP53*	17:7577157	c.783-2A>C		Splice site	8.79
180	*TP53*	17:7578495	c.433_435del	p.Leu145del	Frameshift del	19.3
184	*TP53*	17:7577120	c.818G>A	p.Arg273His	SNV; missense	11.06
25	*ASXL1*	20:31022442	c.1934dup	p.Gly646TrpfsXaa12	Frameshift ins	28.64
115	*ASXL1*	20:31022442	c.1934dup	p.Gly646TrpfsXaa12	Frameshift ins	22.7
125	*ASXL1*	20:31022442	c.1934dup	p.Gly646TrpfsXaa12	Frameshift ins	27.72
130	*ASXL1*	20:31023408	c.2893C>T	p.Arg965Xaa	SNV; missense	9.03
196	*ASXL1*	20:31022793	c.2278C>T	p.Gln760Xaa	SNV; nonsense	38.79
61	*DNMT3A*	2:25457242	c.2645G>A	p.Arg882His	SNV; missense	15.69
128	*DNMT3A*	2:25457242	c.2645G>A	p.Arg882His	SNV; missense	37.12
198	*DNMT3A*	2:25457278	c.2603_2609del	p.Phe868SerfsXaa11	Frameshift del	23.97
57	*IDH2*	15:90631934	c.419G>A	p.Arg140Gln	SNV; missense	46.03
193	*IDH2*	15:90631934	c.419G>A	p.Arg140Gln	SNV; missense	19.22
101	*ZRSR2*	X:15821921	c.312+2T>A		Frameshift ins	10.98
104	*ZRSR2*	X:15838370	c.868C>T	p.Arg290Xaa	SNV; nonsense	92.73
61	*ETV6*	12:12006434	c.403del	p.His135ThrfsXaa74	Frameshift del	16.02
196	*PHF6*	X:133511706	c.59_60insT	p.Lys21Xaa	Frameshift ins	74.96
106	*SRSF2*	17:74732959	c.286G>A	p.Pro95Leu	SNV; missense	35.29
213	*EZH2*	7:148514322	c.1402T>G	p.Cys468Gly	SNV; missense	20.77
99	*CSF3R*	1:36932076	c.2474G>A	p.Gly825Glu	SNV; missense	39.63

Supplemental Table 2. Baseline clinical characteristics in patients with and without non-MPN driver mutations (NDM).

Differences were analyzed using the chi-squared test for categorical variables or fisher's exact test if a value less than 5 in any cells of the contingency table and non-parametric Mann-Whitney U test for continuous variables (i.e. age, disease duration, blood counts). MPN=myeloproliferative; patients=patients; n=number; BAT=best available therapy; Hb=hemoglobin; HC=hydroxycarbamide; Hct=hematocrit; NPM=non-MPN (myeloproliferative neoplasm) driver mutation; P32=radioactive phosphorus; Plt: platelet count; RUX=ruxolitinib; y=years; TN=triple negative; WBC=white blood cell count.

Baseline Clinical Characteristics		Patients with NDM n (%)	Patients without NDM n (%)	*P*
All patients (n=110)		33 (30)	77 (70)	
Median age in years (range)		71 (44 – 91)	64 (35 – 85)	0.0001
Gender	**Female** **Male**	17 (51.5) 16 (48.5)	49 (63.6) 28 (36.4)	0.234
HC-Resistant **HC-Intolerant**		19 (57.6) 14 (42.4)	34 (44.2) 43 (55.8)	0.197
Treatment	**BAT** **RUX**	17 (51.5) 16 (48.5)	35 (45.5) 42 (54.5)	0.560
Disease Duration at TE (y)		6.6 (0.8 – 31)	7.7 (0.4 – 25.9)	0.699
No. of Prior Therapy Lines	**<3** **³3**	27 (81.8) 6 (18.2)	52 (67.5) 25 (32.5)	0.127
Interferon **Anagrelide**		2 (6.1) 15 (45.5)	16 (20.8) 38 (50.6)	0.089 0.706
Busulfan/P32/Pipobroman		3 (9.1)	9 (11.7)	1.0
Previous Thrombosis **Previous Hemorrhage**		7 (21.2) 2 (6.1)	28 (36.4) 4 (5.2)	0.118 0.855
Baseline palpable spleen		3 (9.1)	7 (9,1)	1.0
Baseline blood counts (median, range)	**WBC (x 10^9/l)** **Hb (g/l)** **Hct (%)** **Plt (x10^9/l)**	5.8 (1.7 – 15.2) 115 (90 – 147) 36 (28 - 45) 517 (166 – 1406)	6.1 (2.6 – 29.8) 125 (87 – 160) 38 (27 – 49) 530 (89 – 1139)	0.917 0.01 0.207 0.927
Driver mutation status	***JAK2*V617F** ***CALR*** ***MPL*** **TN**	19 (57.6) 7 (21.2) 3 (9.1) 4 (12.1)	36 (46.8) 26 (33.8) 2 (2.6) 13 (16.9)	0.237
***JAK2*V617F allele burden ≥50%**		5/19 (26.3)	6/36 (16.7)	0.395
***CALR* allele burden ≥50%**		0/7 (0)	2/26 (7.7)	1.0

Supplemental Table 3. Logistic regression predicting the influence of non-MPN driver mutations (NDM) on clinical outcomes. All models were adjusted for *JAK2*V617F mutation status and treatment type since patients were stratified by these at trial entry. Further adjusted analysis was performed for outcomes with significant ($p<0.05$) odd ratios (OR) on initial analysis to include age, TE hemoglobin level and platelet counts denoted indicated with "(adj)" next to OR. The presence of a HMR mutation significantly increased the odds of a transformation event, p=0.015. Hemorrhagic events outcomes were associated specifically with the presence of SF mutations. Platelet counts closest to the hemorrhagic event were normal in 3 of these SF-mutated patients and reduced (*ZRSR2*- mutated; 54 x 10^9/l) and elevated (*SF3B1*-mutated, 504 x 10^9/l) in one patient each. adj=adjusted; AML=acute myeloid leukemia; CHR=complete hematological response; CI=confidence interval; ELN; European LeukemiaNet; HMR=high molecular risk mutation (SF and/or *TP53* mutations); MF=myelofibrosis; n_E =number of events; non-MPN (myeloproliferative neoplasm) driver mutation; OR=odds ratio; RUX=ruxolitinib; SF= splicing factor mutation (*SF3B1, ZRSR2, SRSF2)*; TE=trial entry; UV=univariate. *Driver mutation allele burden ³50% and gender also included in final model for transformation.

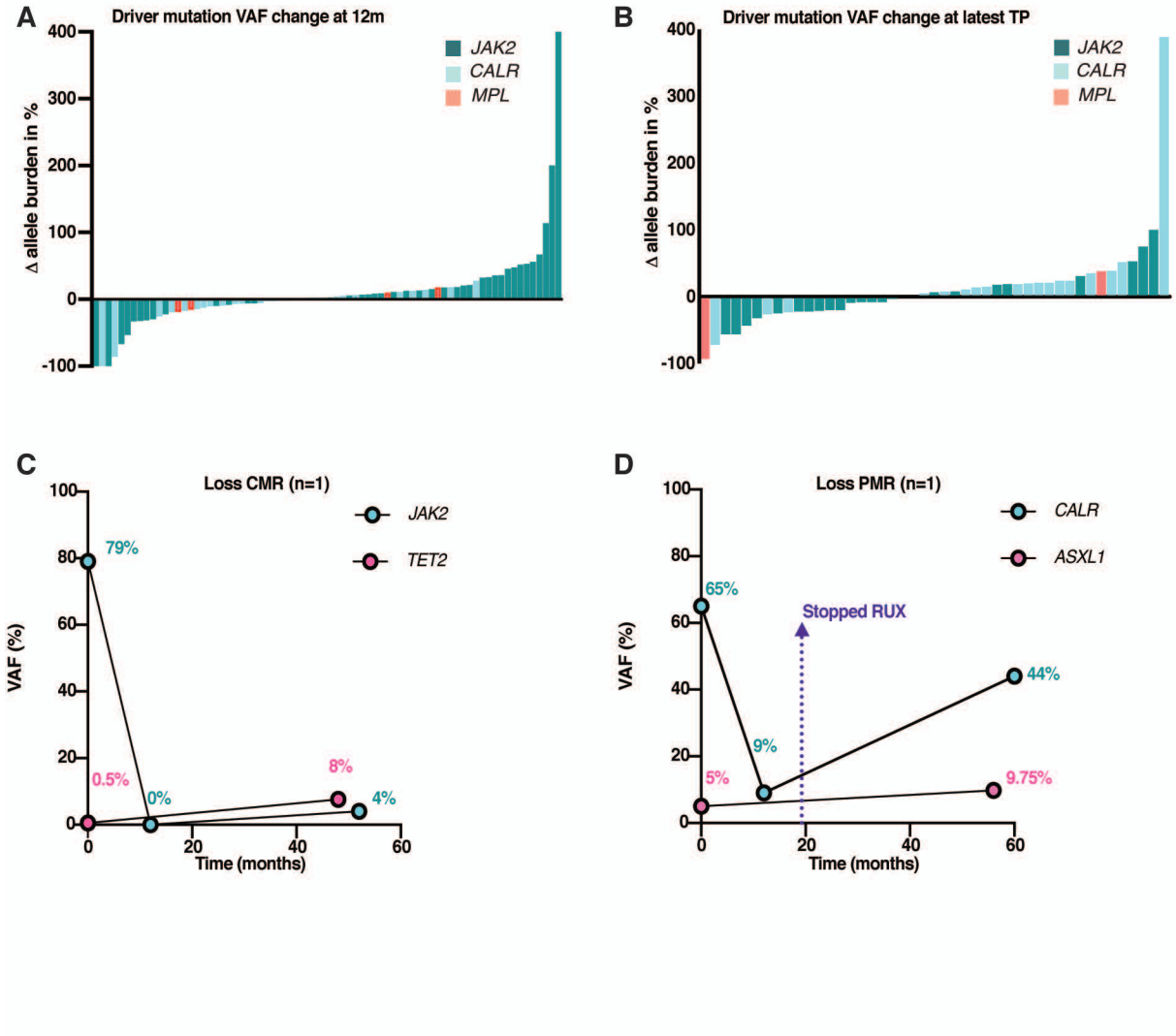
Outcome (n_E)	OR (UV)	OR 95% CI	*P*
1-year CR (ELN) (n_E=50)	0.72	0.3 – 1.6	0.43
1-year post hoc platelet response (n_E =55)	0.5	0.2 – 1.2	0.12
1-year Overall symptom score response (³50% reduction) (n_E =12)	0.71	0.2 – 2.6	0.61
Transformations (MF n_E =13, AML n_E =1)	3.8 (NDM) (adj)* 14.4 (HMR) (adj)*	0.7– 21.5 1.7-122.8	0.129 0.015
Thrombotic event (n_E =21)	0.75	0.2 – 2.3	0.62
Hemorrhagic event (n_E =10)	2.1 (NDM)(adj) 18.9 (SF)(adj)	0.5 – 10 2.2 - 161	0.34 0.007
Death (n_E =13)	2.14	0.6– 2.3	0.21
Stopping RUX treatment (n_E =40)	0.38 (adj)	0.7 – 1.9	0.25

Supplemental Table 4. List of new follow-up NDM including genomic position, nucleotide change, protein consequence, mutation type and variant allele frequency (VAF).

ID	Gene	Genomic position	Nucleotide change	Protein consequence	Mutation type	VAF (%)
55	*TET2*	4:106158481	c.3382dup	p.Tyr1128LeufsXaa2	Frameshift ins	16.68
55	*TET2*	4:106162529	c.3443A>G	p.Tyr1148Cys	SNV; missense	17.29
56	*TET2*	4:106157914	c.2815C>T	p.Gln939Xaa	SNV; nonsense	7.63
118	*TET2*	4:106190831	c.4109G>A	p.Gly1370Glu	SNV; missense	6.15
180	*TET2*	4:106155238	c.141_150del	p.Val48ThrfsXaa16	Frameshift del	6.79
203	*TET2*	4:106164080	c.3590A>G	p.Lys1197Arg	SNV; missense	5.97
55	*ASXL1*	20:31023440	c.2925T>A	p.Cys975Xaa	SNV; nonsense	13.61
55	*ASXL1*	20:31022442	c.1934dup	p.Gly646TrpfsXaa12	Frameshift ins	17.73
67	*ASXL1*	20:31022442	c.1934dup	p.Gly646TrpfsXaa12	Frameshift ins	9.75
76	*ASXL1*	20:31022903	c.2388G>A	p.Trp796Xaa	SNV; nonsense	23.25
89	*TP53*	17:7578206	c.643A>G	p.Ser215Gly	SNV; missense	14.5
128	*TP53*	17:7577568	c.713G>A	p.Cys238Tyr	SNV; missense	6.67
128	*TP53*	17:7579515	c.169_172del	p.Asp57GlnfsXaa65	Frameshift del	7.43
81	*SF3B1*	2:198267359	c.1998G>C	p.Lys666Asn	SNV; missense	5.77
118	*SF3B1*	2:198267359	c.1998G>C	p.Lys666Asn	SNV; missense	5.44
114	*SETBP1*	18:42531907	c.2602G>A	p.Asp868Asn	SNV; missense	36.44
128	*U2AF1*	21:44524456	c.101C>T	p.Ser34Phe	SNV; missense	8.5

Supplemental Figure 1. (A) Waterfall plot of driver mutation change in VAF for each patient at 12months; median change 15.3% (0-400%) and (B) driver mutation change in VAF from 12m to latest time point; median 21.6% (0-389%). (C) BAT-treated patient achieving a JAK2 V617F CMR at 12 months; VAF 22 to 0%. Subsequently, they had a loss of CMR at 44 months with JAK2 V617F VAF 4% coinciding emergence of low level TET2 mutation. (D) RUX-treated patient achieving a CALR PMR at 12 months; VAF 65 to 9%. Subsequently, they had a loss of PMR with a CALR VAF 44% at 60 months and antecedent to this, an ASXL1 mutation emerged at 56 months. Notably, this patient switched from RUX at 20 months due to toxicity. BAT=best available therapy; CMR=complete molecular response; VAF=variant allele frequency; RUX=ruxolitinib; PMR=partial molecular response; TE=trial entry; n=number.

Supplemental Figure 1.



Supplemental Methods

Inclusion criteria and outcome measures were previously reported.[1,2] Peripheral blood (PB) and bone marrow (BM) samples were collected from all patients at baseline and serially, PB samples every 3-4 months and BM samples annually during the study period. Genomic DNA (gDNA) was isolated using DNeasy Blood and Tissue Kit (Qiagen) as per manufacturer's instructions from either PB whole blood or stored PB granulocyte pellets. Driver mutations were sequenced using a next generation sequencing assay as previously described.[3] Mutational profiles of NDM were analyzed using an International Organization for Standardization Standardization (ISO 15189:2012) accredited Illumina TruSeq Custom Amplicon Panel including 32 gene mutation hotspots & exons frequently mutated in myeloid malignancies (~56,000 bp, 341 amplicons); *ASXL1, ATRX, DNMT3A, EZH2, TET2, CEBPA, ETV6, NPM1, PHF6, RUNX1, SETBP1, SF3B1, SRSF2, TP53, U2AF1, WT1, ZRSR2, CBL, CBLB, CBLC, CSF3R, FLT3, HRAS, JAK2, KIT, KRAS, MPL, NRAS, PDGFRA, PTEN, IDH1, IDH2.*[4] Paired-end indexed libraries were prepared for each patient and sequenced on the Illumina MiSeq platform. FASTQ files were aligned to the reference genome (GRCh37/hg19). The minimum depth was ≥100 reads per base and minimum accepted coverage was achievement of this depth in ≥95% of targeted bases. Acceptable coverage was achieved in 99.1% (109/110) of patients. Alignment and variant calling were performed in Basespace (utilizing BWA and GATK/Somatic variant caller; Illumina), while filtering and annotation were performed using a combination of Variant Studio (Illumina) and a custom designed in-house algorithm to evaluate the variants identified. Pathogenic significance of each variant was determined using Exome Aggregation Consortium (ExAC) population frequencies, the Single Nucleotide Polymorphism database (dbSNP), the Catalogue Of Somatic Mutations In Cancer (COSMIC) databases and published literature. Variants considered pathogenic included those reported as somatic in COSMIC and not found in germline databases, those present at the same genomic location of a somatic mutation reported in COSMIC, those not reported in dbSNP databases with a variant allele frequency (VAF) <40-45% but predicted to result in a truncated protein. Single-nucleotide variants (SNVs) were excluded if they had a population frequency of >1% or had a population frequency of <1% but with ethnicity bias and a variant allele frequency (VAF) close to 50%.

Clinical characteristics were correlated with mutations using Pearson's chi-squared test, Student's t-test and Mann-Whitney U test. NDM were analyzed as individual mutations and mutation groups (e.g. splicing factor, epigenetic) comparing these to patients without these or "wild-type" (e.g. *TET2*-mutated patients vs. *TET2*-wild type patients). The impact of

specific co-mutations (e.g. patients with *TET2* co-mutated with *TP53* vs. patients *TET2* mutated alone) was not analyzed as the number of events were too small to examine significant associations. Logistic regression was applied to examine the influence of NDM on treatment response and adverse event adjusting for *JAK2*V617F status and treatment as per trial entry (TE) stratification[1]; additional adjustment was performed if NDM were found statistically significant ($p<0.05$). Median follow-up time calculated using the reverse Kaplan-Meier method[5] with the event of interest reversed. Survival outcomes measured included overall survival, transformation-free survival (TFS) and thrombotic/hemorrhagic-free survival using Kaplan-Meier method and log-rank test. Myelofibrosis, leukemia or death were considered events for TFS calculation. Multivariable analysis of survival outcomes was performed using the Cox proportional hazards regression model; including in the model covariates which had a p-value <0.05 on univariate cox regression analysis. Statistical analyses were performed using SPSS version 25 and R statistical software package version 3.4.0[6] with RStudio version 1.1.463. For access to original individual patient clinical data, please contact the corresponding author.

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