Validation and molecular integration of the RR6 model to predict survival after 6 months of therapy with ruxolitinib

The development of JAK inhibitors (JAKis) marked a revolutionary breakthrough in the therapeutic landscape of myelofibrosis (MF).[1,2] Ruxolitinib (Rux) is associated with consistent response in terms of spleen volume, symptoms, and quality of life. However, almost half of the patients lose their response after a median of 3 years, and a minority are primarily refractory.[3] In addition, Rux may present dose-limiting toxicities eventually leading to dose reduction and/or discontinuation. Most importantly, Rux failure is associated with evidence of clonal progression and dismal prognosis,[4-7] with an estimated overall survival (OS) of less than 18 months.[6-8]

The impact of clinical and/or molecular variables on treatment outcomes in MF patients treated with Rux is still a matter of debate. Recently, the RUXO REL-MF study group developed a clinical prognostic model, named "response to Rux after 6 months" (RR6), that allows for the early identification of Rux-treated MF patients with impaired survival.[9] The model includes three predictor variables collected at baseline, 3 and 6 months, and identifies three risk categories with distinct OS. The RR6 model was validated in an independent cohort of 140 patients.[10]

In this retrospective, single-center study, we aimed to validate the RR6 model, compare its performance with currently validated prognostic models, and explore the independent contribution of genetic factors. The study was conducted in accordance with European and Italian regulations, and was approved by the ethical committees of each institution. The study included 105 patients with World Health Organization-defined MF who were treated with Rux at CRIMM (Florence, Italy), fully annotated for clinical and genetic variables, the latter available in 103 of 105 (98%) patients.

Patient characteristics at Rux start are listed in Table 1. All patients were treated with Rux for at least 6 months, with a median treatment time of 28 (range, 6-130) months. Rux dose was <40 mg daily in 74 (70%), 85 (81%), and 87 (83%) patients at baseline, 12 and 24 weeks, respectively. Transfusion need was reported in 16 (15%) patients at all time points and 43 (41%) at 12 and/or 24 weeks. Palpatory spleen reduction ≤30% at 12 and 24 weeks was observed in 34 (32%) patients. A total of 44 (43%) patients harbored at least one high molecular risk mutation (HMR^{mt}; i.e., mutations in *ASXL1, EZH2, IDH1, IDH2, SRSF2,* or *U2AF1*),[11,12] with 12 (12%) having ≥2 HMR^{mt}. Mutation in RAS pathway genes ($RASp^{mt}$; i.e., *NRAS, KRAS, CBL*) were found in nine (9%) patients.

After a median follow-up of 86 (range, 71-109) months, 38 (36%) patients were still on treatment. Sixty-seven (64%) discontinued Rux, with most frequent reasons for discontinuation including death (27%), resistance (15%), hematological toxicity (13%), and hematopoietic stem cell transplantation (10%). According to the RR6 model, 17 (16%), 50 (48%), and 38 (36%) patients were classified as low (LoR), intermediate (InR), and high risk (HiR), respectively. The estimated median OS from 6 months after Rux start was not reached (NR) (95% confidence interval [CI]: 49-NR), 66 (95% CI: 34-135), and 22 (95% CI: 21-35) months, respectively in the three risk categories (P<0.0001; Figure 1). Although HiR patients had a significant higher risk of death compared to both InR (hazard ratio [HR]=2.8; 95% CI: 1.6-4.9; P=0.0003) and LoR (HR=5; 95% CI: 2-12.2; P=0.0005) patients, the latter two showed a not significantly different outcome. These findings, while overall validating the RR6 model, raise concerns regarding its capability to effectively discriminate lower risk patients. Blast transformation was reported in no, three (6%), and ten (26%) patients in the RR6 LoR, InR, and HiR categories, respectively (P=0.0039).

Next, we investigated whether the RR6 model provided more accurate prognostic information than other currently validated, dynamic prognostic models, such as the Dynamic International Prognostic Scoring System (DIPSS). Overall, RR6 risk categories were broadly represented across the baseline DIPSS ($DIPSS^{bl}$) ones, especially for LoR and HiR (*Online Supplementary Figure S1A*). However, a more heterogeneous composition was observed in $DIPSS^{bl}$ In-1R and In-2R categories, that were enriched in RR6 HiR and LoR patients, respectively. Actuarial survival curves according to $DIPSS^{bl}$ are reported in *Online Supplementary Figure S1B*; while the $DIPSS^{bl}$ reliably discriminated lower risk patients, the OS of HiR and In-2R patients did not differ significantly. Aimed to compare the predictive performance of the RR6 *versus* $DIPSS^{bl}$ models, we computed the respective C-index, Brier score, and time-dependent area under the curve (AUC) (Figure 2A-C). Overall, the RR6 model proved to be superior at all time points. Further, we investigated how the DIPSS prognostic performance changed along Rux treatment by recomputing the score at week 24 ($DIPSS^{w24}$). Among 104 evaluable patients, 35 (34%) and 18 (17%) switched to a lower and higher risk category, respectively (*Online Supplementary Figure S1C*). However, the statistical performance of $DIPSS^{w24}$ did not improve (Figure 2A-C).

Then, we investigated the contribution of genetic variables, in particular conventional cytogenetics (available in 92/105 patients), driver and additional mutations. Median time between cytogenetic/molecular studies and Rux initiation was 5.7 (range, 0.2-68.8) and 4.9 (range, 0-145.4) months, respectively. Univariate Cox proportional hazards analysis identified the following molecular signatures as being associated with inferior OS (*Online Supplementary Table S1*):

Table 1. Characteristics of patients at ruxolitinib initiation in the whole cohort and according to the RR6 model.

Variable	All patients N=105	RR6 low risk N=17 (16%)	RR6 intermediate risk, N=50 (48%)	RR6 high risk N=38 (36%)	*P*
Clinical and demopraphics					
WHO 2016 diagnosis, N (%)					0.7
Pre-PMF	7 (7)	2 (1)	4 (8)	1 (3)	
Overt PMF	43 (41)	7 (41)	19 (38)	17 (45)	
Post-PV/ET MF	55 (52)	8 (47)	27 (54)	20 (53)	
Male sex, N (%)	50 (48)	7 (41)	22 (44)	21 (55)	0.5
Age in years, median (range)	66 (36-88)	58 (38-77)	66 (36-82)	67 (49-88)	0.1
Age >65 years, N (%)	55 (52)	6 (35)	27 (54)	22 (58)	0.3
Leukocytes x10^9/L, median (range)	12.5 (2.5-80)	11.2 (4.3-37.4)	13.2 (2.6-80)	12.1 (2.5-46.1)	0.9
Leukocytes >25x10^9/L, N (%)	25 (24)	6 (35)	10 (20)	9 (24)	0.4
Hemoglobin g/dL, median (range)	11.1 (6.5-16.7)	12.6 (10.3-14.4)	11.2 (8.1-15.2)	9.1 (6.5-16.7)	0.0002
Hemoglobin <10 g/dL, N (%)	38 (36)	0 (0)	13 (26)	25 (66)	<0.0001
RBC transfusion dependence, N (%)	18 (17)	0 (0)	0 (0)	18 (47)	<0.0001
Platelets x10^9/L, median (range)	193 (38-1,114)	304 (165-530)	183 (38-729)	170 (53-1,114)	0.0016
Platelets <100x10^9/L, median (range)	20 (19)	0 (0)	11 (22)	9 (24)	0.1
PB blasts %, median (range)	0 (0-10)	0 (0-2)	0 (0-8)	1 (0-10)	0.1
PB blasts ≥1%, N (%)	41 (40)	5 (29)	16 (33)	20 (53)	0.1
PB blasts ≥2%, N (%)	31 (30)	2 (12)	14 (29)	15 (39)	0.1
BM fibrosis grade ≥2, N (%)	96 (91)	15 (88)	45 (90)	36 (95)	0.6
Palpable spleen below the LCM, cm (range)	15 (1-33)	13 (1-29)	15 (4-26)	16 (5-33)	0.1
Constitutional symptoms, N (%)	77 (73)	14 (82)	37 (74)	26 (68)	0.6
Prognostic stratification, N (%)					
DIPSS risk stratification					0.0003
Low risk	3 (3)	2 (12)	1 (2)	0 (0)	
Intermediate-1 risk	40 (38)	4 (24)	15 (30)	21 (55)	
Intermediate-2 risk	51 (49)	11 (65)	31 (62)	9 (24)	
High risk	11 (10)	0 (0)	3 (6)	8 (21)	
MPN drivers					
JAK2 mutated, N (%)	81 (77)	14 (82)	40 (80)	27 (71)	0.5
JAK2^{V617F} AB, median (range), ev N=81	71 (12-100)	58 (31-90)	77 (32-100)	72 (12-99)	0.3
CALR mutated, N (%)	19 (19)	2 (13)	7 (15)	10 (28)	0.3
MPL mutated, N (%)	4 (4)	2 (13)	1 (2)	1 (3)	0.2
Triple negative, N (%)	2 (2)	0 (0)	2 (4)	0 (0)	0.3
Myeloid neoplasm-associated genes, N (%)					
ASXL1 mutated, ev N=103	37 (36)	3 (18)	20 (41)	14 (38)	0.2
CBL mutated, ev N=100	2 (2)	0 (0)	0 (0)	2 (6)	0.1
CSF3R mutated, ev N=86	0 (0)	0 (0)	0 (0)	0 (0)	-
CUX1 mutated, ev N=79	0 (0)	0 (0)	0 (0)	0 (0)	-
DNMT3A mutated, ev N=99	3 (3)	0 (0)	3 (6)	0 (0)	0.1
EZH2 mutated, ev N=103	10 (10)	1 (6)	5 (10)	4 (11)	0.8
IDH1/2 mutated, ev N=103	4 (4)	2 (12)	1 (2)	1 (3)	0.2
KIT mutated, ev N=98	0 (0)	0 (0)	0 (0)	0 (0)	-
KRAS mutated, ev N=97	7 (7)	2 (13)	3 (7)	2 (6)	0.7
NF-E2 mutated, ev N=90	11 (12)	0 (0)	6 (13)	5 (17)	0.3
NRAS mutated, ev N=97	11 (11)	2 (13)	4 (9)	5 (14)	0.7
PTPN1 mutated, ev N=86	3 (3)	1 (8)	1 (2)	1 (3)	0.7
RUNX1 mutated, ev N=98	6 (6)	1 (6)	2 (4)	3 (9)	0.7
SETBP1 mutated, ev N=86	1 (1)	0 (0)	1 (2)	0 (0)	0.6
SF3B1 mutated, ev N=99	5 (5)	2 (13)	1 (2)	2 (6)	0.3
SH2B3/LNK mutated, ev N=99	10 (10)	2 (13)	4 (9)	4 (11)	0.9
SRSF2 mutated, ev N=103	6 (6)	1 (6)	2 (4)	3 (8)	0.7
TET2 mutated, ev N=100	25 (25)	4 (25)	9 (19)	12 (33)	0.3
TP53 mutated, ev N=98	1 (1)	0 (0)	1 (2)	0 (0)	0.6
U2AF1 mutated, ev N=103	1 (1)	0 (0)	0 (0)	1 (3)	0.4
ZRSR2 mutated, ev N=86	9 (10)	1 (8)	3 (7)	5 (16)	0.4
HMR mutations,† ev N=103	44 (43)	5 (29)	23 (47)	16 (43)	0.4
≥2 HMR mutations, ev N=103	12 (12)	2 (12)	5 (10)	5 (14)	0.9
≥1 RASp mutation,‡ ev N=99	9 (9)	2 (13)	3 (6)	4 (11)	0.7

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Variable	All patients N=105	RR6 low risk N=17 (16%)	RR6 intermediate risk, N=50 (48%)	RR6 high risk N=38 (36%)	*P*
Cytogenetics, N (%)					
Conventional two-tiered cytogenetic, ev N=92					0.1
Favorabe karyotype	76 (83)	13 (93)	39 (89)	24 (71)	
Unfavorable karyotype	16 (17)	1 (7)	5 (11)	10 (29)	
Revised three-tiered cytogenetic, ev N=92					0.3
Favorable karyotype	66 (72)	11 (79)	34 (77)	21 (62)	
Unfavorable karyotype	21 (23)	3 (21)	9 (20)	9 (26)	
Very high-risk karyotype	5 (5)	0 (0)	1 (2)	4 (12)	
RR6 model,N (%)					
Rux dose <40 mg daily at all time points	69 (66)	0 (0)	32 (65)	37 (97)	<0.0001
Rux dose <40 mg daily at baseline	74 (70)	0 (0)	36 (72)	38 (100)	<0.0001
Rux dose <40 mg daily at 12 weeks	85 (81)	5 (29)	42 (84)	38 (100)	<0.0001
Rux dose <40 mg daily at 24 weeks	87 (83)	8 (47)	42 (84)	37 (97)	<0.0001
RBC transfusion need all time points	16 (15)	0 (0)	0 (0)	16 (42)	<0.0001
RBC transfusion need at 12 and/or 24 weeks	43 (41)	0 (0)	21 (42)	22 (58)	0.0003
Splenomegaly reduction ≤30% at 12 and 24 weeks	34 (32)	0 (0)	3 (6)	31 (82)	<0.0001
Splenomegaly reduction <30% at 12 weeks	46 (44)	0 (0)	13 (26)	33 (87)	<0.0001
Splenomegaly reduction <30% at 24 weeks	40 (38)	0 (0)	7 (14)	33 (87)	<0.0001

Notes: †HMR mutations include pathogenic variants in any of the following genes: *ASXL1, EZH2, IDH1, IDH2, SRSF2* or *U2AF1*; ≥2 HMR mutations indicates the presence of 2 or more mutations (2 or more mutations in the same gene are counted as 1). ‡RAS pathway mutations include pathogenic variants in any of the following genes: *NRAS, KRAS*, and *CBL*. A varinat allele frequency (VAF) of 2% was used as threshold value for somatic variants. AB: allele burden; BM: bone marrow; DIPSS: Dynamic International Prognostic Score System; ET: essential thrombocythemia; HMR: high molecular risk mutation; LCM: left costal margin; MF: myelofibrosis; MPN: myeloproliferative neoplasm; PB: peripheral blood; PMF: primary myelofibrosis; Pre-PMF: prefibrotic-PMF; PV: polycythemia vera; RASp: RAS pathway; RBC: red blood cell; RR6: response to ruxolitinib after 6 months; Rux: ruxolitinib; WHO: World Health Organization; ev: evaluable.

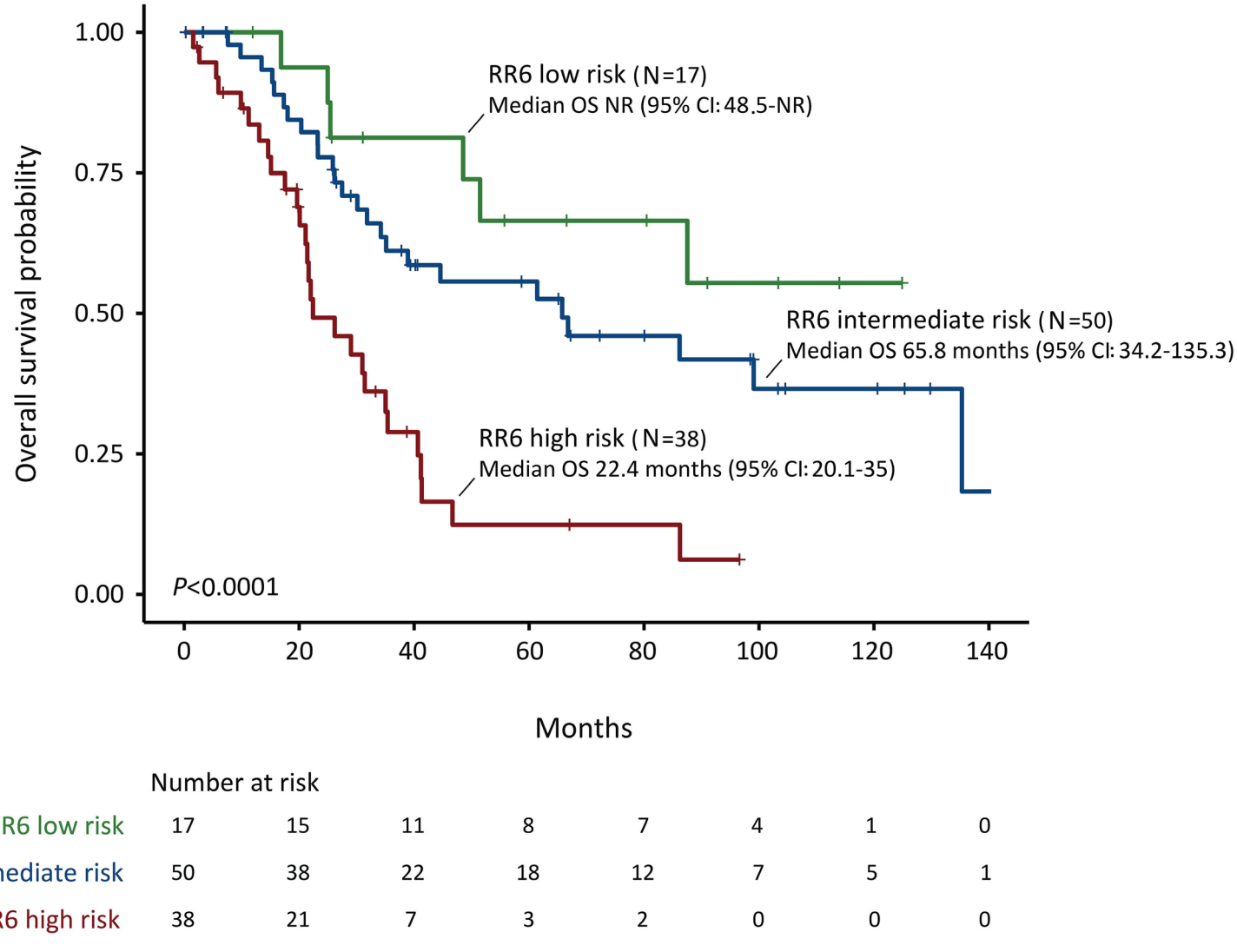


Figure 1. Kaplan-Meier estimates of overall survival in ruxolitinib-treated patients according to the RR6 model. CI: confidence interval; NR: not reached; OS: overall survival; RR6: response to ruxolitinib after 6 months.

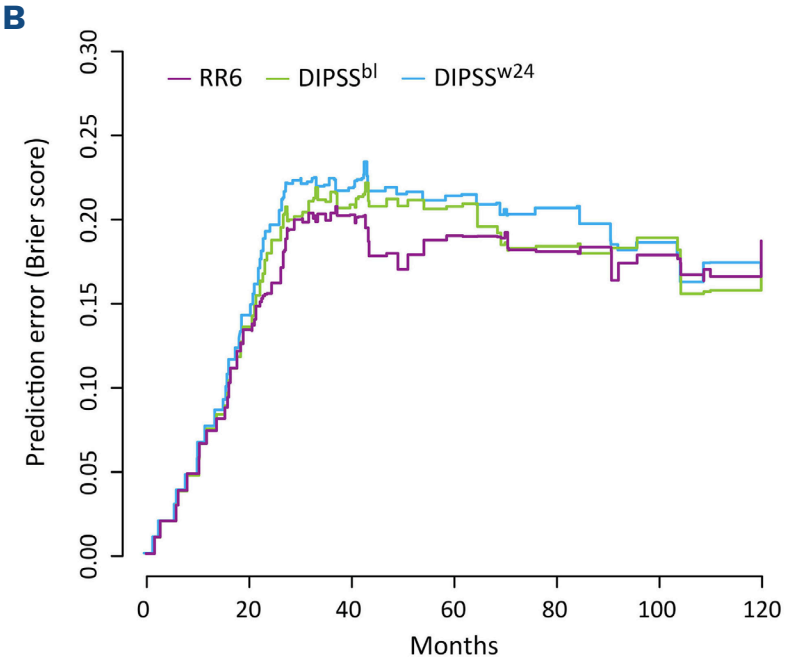
unfavorable karyotype according to the conventional two-tiered cytogenetic risk model,[13] *ASXL1*^{mt}, *SRSF2*^{mt}, harboring ≥1 HMR^{mt}, and having RASp^{mt}. Upon multivariate analysis, RR6 (HiR *vs*. InR: HR=3.1; 95% CI: 1.7-5.9; *P*=0.0004; HiR *vs*. LoR: HR=4.4; 95% CI: 1.7-11.1; *P*=0.0020), unfavorable karyotype (HR=3.2; 95% CI: 1.5-6.7; *P*=0.0019), ≥1 HMR^{mt} (HR=2.5; 95% CI: 1.4-4.6; *P*=0.0023), and RASp^{mt} (HR=6.1; 95% CI: 2.2-17; *P*=0.0005) remained independent predictors of reduced OS. Next, we evaluated the prognostic contribution of genetic features by computing the C-index, Brier score, and AUC of the RR6 after its integration with HMR^{mt} and/or RASp^{mt} (Figure 2A, D, E). The highest values for performance and accuracy were achieved by the RR6-HMR^{mt}-RASp^{mt} combination, that showed to be superior at all time points, followed by the RR6-RASp^{mt} and RR6-HMR^{mt} combinations. These findings were validated using the original RUXO REL-MF cohort. Among the 71 molecularly-annotated patients, 23 (32%) harbored an HMR^{mt}, whereas seven (10%) had a RASp^{mt}. Median time on Rux was 28 (range, 6-93) months. Also in this validation series, the RR6-HMR^{mt}-RASp^{mt} combination had the highest

A

Comparison of the prognostic performance of RR6 model, DIPSS, and RR6 integration with high molecular risk signatures

		Events at 12 months		Events at 24 months		Events at 36 months		Events at 48 months	
	C-index	Brier score	AUC	Brier score	AUC	Brier score	AUC	Brier score	AUC
RR6	66.0	0.033	73.4	0.078	75.9	0.117	72.2	0.135	80.0
DIPSS	65.7	0.033	71.9	0.081	68.0	0.123	71.2	0.145	70.2
DIPSS	63.4	0.034	61.1	0.085	60.5	0.131	68.9	0.154	68.9
HMR^{mt†}	58.2	0.035	61.0	0.084	62.6	0.129	61.6	0.154	61.0
RASp^{mt‡}	54.1	0.034	58.9	0.082	57.5	0.127	56.3	0.153	56.3
RR6+HMR^{mt}	68.7	0.033	77.5	0.077	80.4	0.115	76.0	0.131	82.7
RR6+RASp^{mt}	68.6	0.032	79.3	0.075	80.9	0.112	75.2	0.129	83.3
RR6+HMR^{mt}+RASp^{mt}	**70.5***	**0.032***	**80.9***	**0.074***	**83.5***	**0.10***	**77.8***	**0.126***	**84.8***

B



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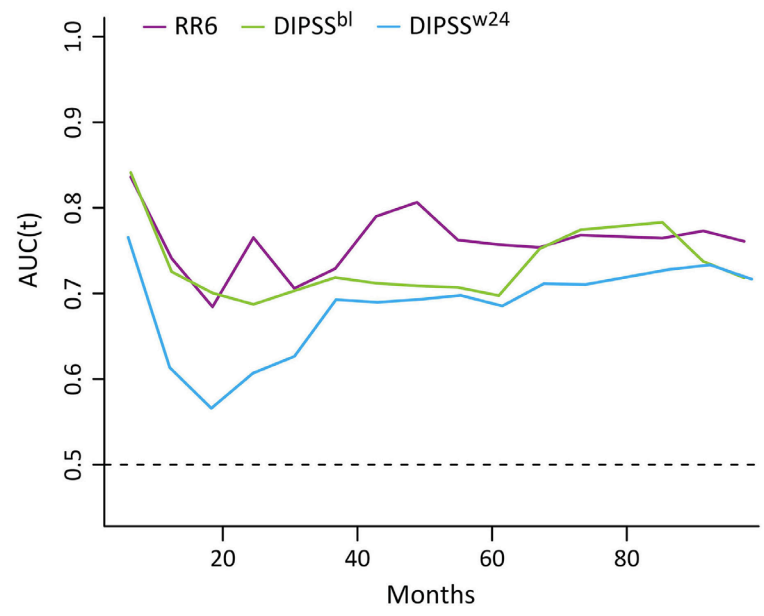

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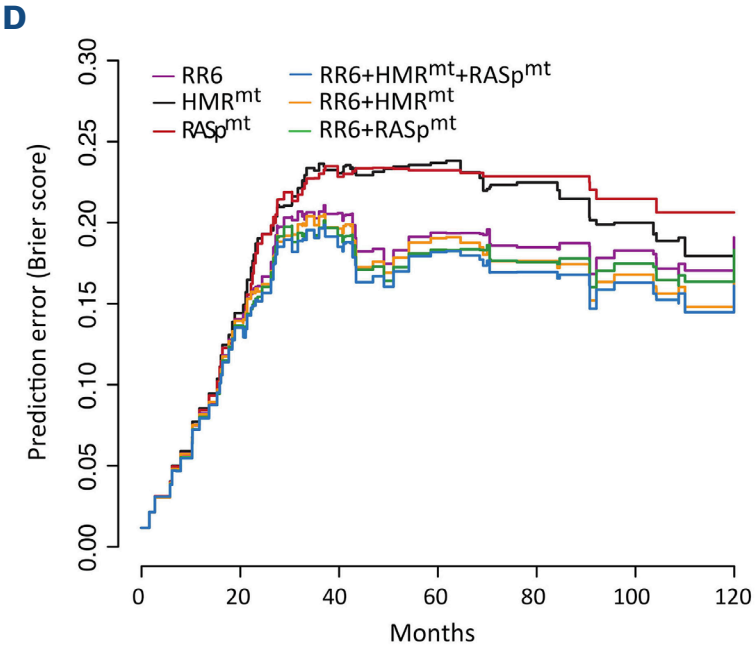

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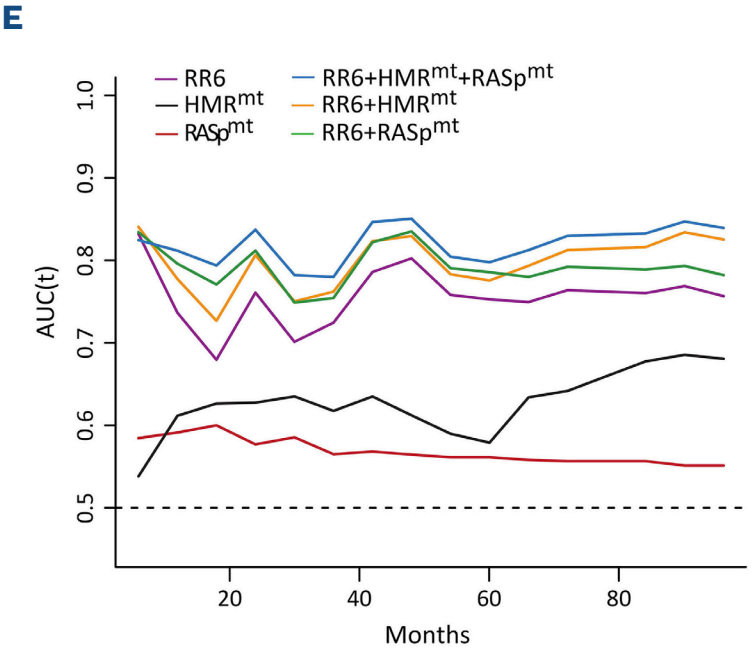

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F

Comparison of the prognostic performance of RR6 model and its integration with high molecular risk signatures in the validation cohort (76 patients of the original RUXO REL-MF cohort)

		Events at 12 months		Events at 24 months		Events at 36 months		Events at 48 months	
	C-index	Brier score	AUC	Brier score	AUC	Brier score	AUC	Brier score	AUC
RR6	70.4	**0.16***	82.2	0.19	78.4	0.20	72.5	0.19	72.5
RR6+HMR^{mt}	74.7	0.17	**92.9***	**0.17***	**82.0***	**0.17***	75.9	**0.17***	75.9
RR6+RASp^{mt}	73.0	0.20	80.7	0.20	78.6	0.20	75.7	0.20	75.7
RR6+HMR^{mt}+RASp^{mt}	**75.7***	0.17	91.3	**0.17***	81.3	**0.17***	**79.3***	**0.17***	**79.3***

Figure 2. Prognostic performance of conventional clinical prognostic and molecularly integrated models. (A) Comparison of the prognostic performance of RR6 model, DIPSS, and RR6 integration with high molecular risk signatures. (B) Brier score for prediction of death measured over time for RR6 model, DIPSS^{bl} and DIPSS^{w24}. (C) Time-dependent area under the curve (AUC) for prediction of death for RR6 model, DIPSS^{bl} and DIPSS^{w24}. (D) Brier score for prediction of death measured over time for RR6 model and its integration with HMR^{mt} and/ore RASp^{mt}. (E) Time-dependent AUC for prediction of death for RR6 model and its integration with HMR^{mt} and/ore RASp^{mt}. (F) Comparison of the prognostic performance of RR6 model and its integration with high molecular risk signatures in the validation cohort. Notes: asterisk and bold indicate the best values. †HMR mutations include pathogenic variants in any of the following genes: *ASXL1, EZH2, IDH1, IDH2, SRSF2* or *U2AF1*. ‡RAS pathway mutations include pathogenic variants in any of the following genes: *NRAS, KRAS* or *CBL*. DIPSS^{bl}: Dynamic International Prognostic Scoring System at baseline; DIPSS^{w24}: Dynamic International Prognostic Scoring System at week 24; HMR^{mt}: high molecular risk mutation; RASp^{mt}: RAS pathway mutation; RR6: response to ruxolitinib after 6 months.

values for performance (C-index and in most instances AUC) (Figure 2F). Accuracy values on the other hand were better for the triple combination than the RR6 alone, but the advantage of the triple *versus* double (RR6-HMR^{mt}) combination was lost, likely due to the relatively small number of patients and events, especially within the RASp^{mt} group. In addition, we confirmed the superiority of the HMR^{mt}-RASp^{mt}-integrated RR6 in a cohort of 116 transplant-age patients (≤70 years) resulting from the combination of our and the original RUXO REL-MF cohorts (*Online Supplementary Figure S2*).

In order to further explore the role of genetic factors, we investigated clonal evolution among RR6 risk categories. Of 54 (51%) patients with molecular data at baseline and follow-up (median time from Rux start, 22 months; range, 3-67), 22 (41%) acquired at least one mutation, with most frequent acquisitions involving *ASXL1* (6/22), *KRAS* (4/22), and *NRAS* (3/22). Notably, new mutation acquisition was enriched in RR6 HiR patients (8/17, 47%), as opposed to LoR (1/12, 8%) and InR (8/27, 30%) patients. Furthermore, acquisition of >1 mutation was observed in five HiR patients, compared to none of LoR and InR patients.

Treatment failure to Rux due to resistance (either primary or secondary) or intolerance is associated with adverse prognosis.[6,8] Therefore, timely identification of MF patients with no or suboptimal response to Rux still represents a major therapeutic caveat. This is even more relevant when considering newly available JAKis and the plethora of novel agents in advanced clinical development.

In this study, we validated the RR6 model in a large, single-center cohort of Rux-treated MF patients with extensive clinical and molecular data. The RR6 model effectively identifies Rux-treated patients with dismal survival, providing a greater prognostic performance compared to the DIPSS. However, our data suggest that the RR6 model may present inferior performance in discriminating lower-risk patients, possibly due to the smaller study cohort. Most importantly, we provided compelling data supporting the role of distinct molecular signatures as additional, independent risk factors. The adverse prognostic role of HMR^{mt} is currently well defined in MF.[11,12,14] In addition, we recently reported that RASp^{mt} are associated with adverse survival outcomes, and may predict reduced response to JAKis.[7] Accordingly, the integration of both HMR^{mt} and RASp^{mt} in the RR6 model remarkably enhanced the performance of the score. We validated these findings in 71 molecularly annotated patients of the original RUXO REL-MF cohort, albeit with some limitations due to the small number. Finally, we showed that clonal evolution is more frequent in patients with RR6-defined HiR disease, thus corroborating the role of genomic instability in Rux response/resistance and disease outcome. Notably, the observation that new mutation acquisition mostly involved *ASXL1*, *KRAS*, and *NRAS* further underscores their significance as key biological drivers in MF.

In conclusion, our findings suggest that i) the RR6 model effectively allows the identification of HiR patients, but suffers from inferior performance in discriminating lower risk patients; ii) integration with HMR^{mt} and RASp^{mt} improves the performance of the score; and iii) in RR6 higher risk patients, inferior survival is pathogenetically associated with clonal evolution.

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Disclosures

No conflicts of interest to disclose.

Contributions

AMV, GCa, GCo and PG designed the research, interpreted the results and wrote/edited the final manuscript. AA, FM, GCa, GCo, MB and ME collected data. CM, EN and LS assisted in performing the molecular research. GCa and GCo performed statistical analysis. MM, BM, AI, MF, MC, LB and FP contributed to the validation analysis using the original RUXO REL-MF cohort and the critical revision of the manuscript for important intellectual content. All authors read and approved the final draft of the manuscript.

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Data-sharing statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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