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ORIGINAL ARTICLE

Effect of MAPK activation via mutations in *NRAS*, *KRAS* and *BRAF* on clinical outcome in newly diagnosed multiple myeloma

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Abstract

Until now, next generation sequencing (NGS) data has not been incorporated into any prognostic stratification of multiple myeloma (MM) and no therapeutic considerations are based upon it. In this work, we correlated NGS data with (1) therapy response and survival parameters in newly diagnosed multiple myeloma, treated by VRd * and (2) MM disease stage: newly diagnosed multiple myeloma (ndMM) versus relapsed and/or refractory (relapsed/refractory multiple myeloma). We analyzed 126 patients, with ndMM and relapsed refractory multiple myeloma (rrMM), treated at the University Hospital of Bern (Inselspital). Next generation sequencing was performed on bone marrow, as part of routine diagnostics. The NGS panel comprised eight genes *CCND1*, *DIS3*, *EGR1*, *FAM46C (TENT5C)*, *FGFR3*, *PRDM1*, *TP53*, *TRAF3* and seven hotspots in *BRAF*, *IDH1*, *IDH2*, *IRF4*, *KRAS*, *NRAS*. The primary endpoint was complete remission (CR) after VRd in ndMM, in correlation with mutational profile. Mutational load was generally higher in rrMM, with more frequently mutated *TP53*: 11/87 (13%) in ndMM versus 9/11 (81%) in rrMM (OR 0.0857, $p = 0.0007$). In ndMM, treated by VRd, mutations in MAPK-pathway members (*NRAS*, *KRAS* or *BRAF*) were associated with reduced probability of CR (21/38, 55%), as compared with *wild type NRAS*, *KRAS* or *BRAF* (34/40, 85%; OR 0.2225, $p = 0.006$). *NRAS* c.181C > A (p.Q61K) as a single mutation event showed a trend to reduced probability of achieving CR (OR 0.0912, $p = 0.0247$). Activation of MAPK pathway via mutated *NRAS*, *KRAS* and *BRAF* genes seems to have a negative impact on outcome in ndMM patients receiving VRd therapy. VRd* - bortezomib (Velcade®), lenalidomide (Revlimid®) and dexamethasone.

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Naomi Porret and Ekaterina Rebmann equal contribution; should both be considered as last authors.

KEYWORDS
genetic risk factor, MAPK pathway, multiple myeloma, next generation sequencing, NGS

1 | INTRODUCTION

Despite the development of highly efficient new treatment strategies in multiple myeloma (MM), the disease remains incurable.[1–3] New therapeutic targets are urgently needed to further improve the outcome.[4–14] Before the introduction of daratumumab (anti-CD38 antibody) into first line MM treatment, the proteasome inhibitor (PI) and immunomodulatory imide drugs (IMiDs) based triplet VRd (bortezomib (Velcade®), lenalidomide (Revlimid®) and dexamethasone), followed by high dose chemotherapy and autologous stem cells transplantation (autoHSCT) was standard of care in newly diagnosed multiple myeloma (ndMM) for eligible patients.[15–17] The addition of daratumumab (DVRd regimen) increases the proportion of deeper response (in terms of minimal residual disease persistence) and eventually allows for better progression free survival (PFS).[18,19] However, the benefit of overall survival (OS) for daratumomab inclusion has yet to be proven.[20,21] Despite the efficacy of VRd and especially DVRd combinations, the outcome in ndMM remains heterogeneous and most of patients will eventually relapse.[22–25]

Next-generation sequencing (NGS) is a diagnostic tool for detecting both somatic and germline mutations in MM.[26] Some recent translational NGS-based studies already considered correlations between mutational landscape in MM and clinical outcome.[4,13,27] However, due to heterogeneity of studied populations and different therapy regimens, clear associations between mutational profile and treatment outcome in MM have not been established. Therefore, assessment of prognostic significance of diverse genomic lesions in homogeneously treated MM patients is greatly needed.

We designed this study to correlate mutational profile with therapy response and survival parameters in ndMM, treated by standard PI/IMiDs -based combination, VRd regimen. We also analyzed the difference in mutational landscape between ndMM and relapsed refractory multiple myeloma (rrMM).

2 | MATERIAL AND METHOD

2.1 | Patients

We studied mutational profiles by NGS in bone marrow (BM) samples from 126 patients with ndMM or rrMM who underwent routine BM examination, complemented by NGS analysis at the University Hospital of Bern (Inselspital) between August 2018 and November 2021. Seven patients were excluded from the analysis of primary outcome because of missing clinical data. All patients signed an informed consent form, agreeing to the use of their data for further studies.

Multiple myeloma was diagnosed according to the criteria of International Myeloma Working Group (IMWG) and current European Society for Medical Oncology guidelines (2021).[15] Staging and risk assessment were performed according to Multiple Myeloma International Staging System or Revised International Staging System systems, depending on whether initial cytogenetic data was available.[28,29]

In patients with ndMM, VRd regimen with or without high dose consolidation and autologous stem cell transplantation (autoHSCT) was used as standard first line treatment.[15] Remission was also evaluated according to IMWG criteria.[8,30]

2.2 | NGS and gene panel design

For NGS analysis, plasma cells were separated from fresh BM aspirates using CD138+ magnetic cell sorting with the autoMACS® Pro Separator. DNA was extracted using the QIAamp DNA mini kit® by Qiagen, as previously described.[31]

Next generation sequencing was performed using the Ion S5 platform, with the Torrent Suite software for variant calling. All mutations were curated manually, using publicly available databases as well as the annotation software Alamut™ Visual Plus. Because the sequencing was done on DNA, extracted from selected CD138+ plasma cell compartment, the allele burden was generally high, with many variants presenting variant allele frequency (VAF) close to 50%. We excluded all known benign germline variants. Pathogenic variants with a high germline probability were not either considered for this study. Next generation sequencing analysis was routinely performed for patients with a first diagnosis of MM. For patients with a relapse or progression of the disease, the decision to perform NGS was based on clinical decision by the physician team.

The NGS Panel was developed for routine diagnostic in patients with MM at the University Hospital of Bern, as previously described.[31]

The genes and hotspots were selected according to the frequency of occurrence given in the literature, their prognostic impact, and - for some markers - for their possible function as therapeutic targets.[27,32,33] The NGS panel comprised 15 genes including splice sites or hotspots: *BRAF* (exons 11 and 15), *CCND1*, *DIS3*, *EGR1*, *FAM46C* (*TENT5C*), *FGFR3*, *IDH1* (exon 4), *IDH2* (exon 4), *IRF4* (exon 3), *KRAS* (exons 2 and 3), *MYD88* (*L265P* mutation), *NRAS* (exons 2 and 3) *PRDM1*, *TP53* and *TRAF3*.[31]

2.3 | Statistical analysis

We used R- software version 4.2.0 for the statistical analysis.

For the primary outcome, we assessed frequency tables for each categorical risk factor against remission status “no” complete

remission (CR) versus CR by Fisher's exact test for count data. This analysis was performed only for the patients, diagnosed with ndMM who received VRd regimen as first-line therapy. We used Benjamini-Hochberg's (BH) method for false discovery rate correction (Type I error).[34] Adjusted *p*-values were considered significant at the 5% level and BH adjusted *p*-value were considered significant at an alpha level of 10%.

The secondary outcomes were PFS and OS. Progression free survival and OS were calculated from the start of treatment in ndMM and analyzed by Kaplan-Meier and log-rank methods. *p*-value ≤0.05 was considered as significant. We also assessed PFS and OS by restricted mean survival time difference, which is the difference in average time of survival at a chosen truncation time. The chosen truncation time "τ" (a right censoring) was based on the minimum of the maximum follow-up times available in each respective group (so called minimax) which is the value that makes use of all follow-up information available.

3 | RESULTS

3.1 | Clinical data and patient outcome

We collected data from 126 patients. Patient characteristics are listed in Table 1. Eighty-five patients (67%) presented with ndMM and 41 (33%) with rrMM. We excluded seven patients from the analysis of primary outcome because of missing further clinical data. Eighty-six patients (68%) were males. The median age was 64 years. The median follow-up time was 7 months in ndMM and 17 months for the rrMM. Ten patients out of 85 in the ndMM subgroup (12%), versus 16 out of 41 in the rrMM subgroup (39%) had died at the time of the follow-up.

The percentage of cases with high-risk cytogenetic aberrations was similar in ndMM and rrMM: 22/75 (29%) and 11/34 (32%), respectively.

The VRd regimen was given in 78/85 (92%) of ndMM.[16] Most of those patients 69/78 (88%) received a high dosis consolidation (HD) followed by autoHSCT, and only 9/78 (12%) were not eligible for the intensive treatment, Table 1. All patients, who received HD consolidation, have reached at least a very good partial remission before the autoHSCT. All patients with ndMM were treated by the year 2020 and no daratumumab was given as the first line treatment.

3.2 | NGS results

3.2.1 | General mutation frequency and type

In total 136 mutations were detected by NGS in 87 out of all 126 (69%) cases, Table 2. The median mutation count was one (standard deviation 1.05), the highest number of mutations per sample was five —in two out of the 41 rrMM cases (4.8%). We presented all detected

TABLE 1 Clinical characteristics of patients.

Parameter	ndMM *n* = 85	rrMM *n* = 41
Median age (years)	64 (σ11.11)	63.5 (σ11.12)
Females	26 (30.6%)	14 (34.1%)
Males	59 (69.4%)	27 (65.9%)
Cytogenetic risk[a]		
High risk	22 (25.9%)	11 (26.8%)
Low risk	53 (62.4%)	23 (56.1%)
R-ISS[b]		
I	18 (21.2%)	8 (19.5%)
II	45 (52.9%)	16 (39.0%)
III	15 (17.6%)	14 (34.1%)
CRAB criteria[c]		
Yes	78 (91.8%)	33 (80.5%)
No	7 (8.2%)	5 (12.2%)
Bone marrow infiltration (histopathology)[d]		
<10%	15 (17.6%)	7 (17.1%)
10%–30%	18 (21.2%)	13 (31.7%)
>30%	50 (58.8%)	20 (48.8%)
Type of paraprotein		
IgA	8 (9.4%)	5 (12.2%)
IgG	56 (65.9%)	22 (53.7%)
IgM	0	2 (4.9%)
Type of light chain		
Kappa	57 (67.1%)	21 (51.2%)
Lambda	28 (32.9%)	20 (48.8%)
Both (biclonal)	1 (1.2%)	0
Treatment		
Proteasome based	78 (91.8%)	39 (95.1%)
Other	7 (8.2%)	2 (4.9%)
Median follow up time (month)	7	16
Mean follow up time (month)	12	15
Median survival time (month)	6.5[e]	15[e]

Note: Cytogenetic risk: (1) high-risk: presence of any high risk mutation (del(17p), t(4; 14) or t(14; 16). (2) absence of any high risk mutation. CRAB criteria according to IMWG.

Abbreviation: R-ISS, Revised International Staging System.

[a]information missing for 10 patients in ndMM group and 7 patients in rrMM group.

[b]information missing for 7 patients in ndMM group and 3 patients in rrMM group.

[c]information missing for 3 patients in rrMM group.

[d]information missing for 2 patients in ndMM group and 1 patient in rrMM group.

[e]10 out of 85 in the ndMM group died during the follow-up time and 16 out of 41 in the ndMM group.

TABLE 2 Mutation distribution in patients with diagnosed multiple myeloma (MM).

Mutations	Overall $n = 126$	ndMM $n = 85$	rrMM $n = 41$
No mutation	39 (31%)	28 (22%)	11 (9%)
1 mutation	56 (44%)	43 (34%)	13 (10%)
>1 mutations	31 (25%)	14 (11%)	17 (13%)
KRAS	35 (27%)	21 (17%)	11 (9%)
NRAS	22 (17%)	15 (12%)	7 (6%)
DIS3	17 (13%)	9 (7%)	5 (4%)
TP53	15 (12%)	2 (2%)	9 (7%)
FAM46C	14 (11%)	7 (6%)	6 (5%)
TRAF3	11 (9%)	6 (5%)	3 (2%)
BRAF	10 (8%)	5 (4%)	4 (3%)
FGRF3	4 (3%)	1 (1%)	3 (2%)
IRF4	3 (2%)	0	3 (2%)
IDH1	2 (1.5%)	2 (2%)	0
EGR1	2 (1.5%)	No data	No data
PRDM1	1 (1%)	1 (1%)	0

Abbreviations: ndMM, newly diagnosed multiple myeloma; rrMM, relapsed multiple myeloma.

mutations according to type, VAF and a possible effect on protein function in the supplementary materials, Table S1.

Regarding the frequency, the most commonly mutated genes across all 87 "NGS positives" cases were *KRAS* 35/87 (40%), *NRAS* 22/87 (25%), *DIS3* 17/87 (20%), *FAM46C* 14/87 (16%), *TP53* 15 (17%), TRAF3 11/87 (12%) and *BRAF* 10/87 (11%), Table 2. Mutations in *KRAS* and *NRAS* were mutually exclusive (Figure 1).

In 56/87 (64%) cases, the mutation was a single event, which was the most frequent for *KRAS* 19/56 (34%) and *NRAS* 13/56 (23%). Isolated mutations were also the most common disruption event for both genes 19 of all 35 *KRAS* mutants (54%) and 13/22 for *NRAS* (59%).

3.2.2 | Mutations frequency and distribution among ndMM and rrMM

In ndMM, absence of mutations was documented in 28/85 (33%) cases, a single mutational event was found in 43/85 (51%) cases and >1/sample mutation was detected in 14/85 (16%) patients (1.0 median mutation in ndMM, standard deviation 1.05).

In rrMM, no mutation was found in 11/41 (27%) cases, a single mutation in 13/41 (32%) of cases and more than one mutation in 17/41 (41%) patients (1.0 median mutation in rrMM, standard deviation 1.05). Tested by Wilcox-test and *t*-test, the number of mutations differ significantly between ndMM and rrMM. As expected, the cases of rrMM showed a greater mutational load, as compared to ndMM, with a higher maximum of mutations per sample—five versus three (p-value <0.05) (Figure 2).

Concerning the distribution across ndMM and rrMM cases, the proportion of mutants was similar for *KRAS* 21/85 (25%) in ndMM versus 11/41 (27%) in rrMM; *NRAS* 15/85 (18%) in ndMM versus 7/41 (17%) in rrMM and *DIS3* 9/85 (11%) in ndMM and 5/41 (12%) in rrMM.

The frequency of *TP53* mutations in rrMM was higher as compared to ndMM nine out of 41 (22%), against 2/85 in ndMM ($p < 0.05$), which is in line with previous publications.[35]

In addition, TP53 was significantly more often mutated in rrMM: nine out of 41 (22%), against two out of 85 in ndMM (p-value <0.05). Interestingly, one case presented five different mutations in *TP53*.

Thirteen mutations were found in *FAM46C*, of those, 7/13 (54%) in ndMM and 6/13 (46%) in rrMM, without statistically significant difference (p-value 0.35).

The isolated *NRAS* mutations were most common for ndMM 11/13 (85%) with only 2/13 (15%) being found in rrMM. The most shared was Gly change at position 61 (Q61), found in 16/22 (72%). Among these, the substitution *c*.181C > *A* (*p.Q61*K) was the most frequent 9/22, or 41% of all *NRAS* mutants. Of those, six were found in ndMM (67%) and three in rrMM (33%). Mutation *p.Q61*K in *NRAS* was also the most frequent among all cases with single mutational event 6/56 (11%).

In *KRAS*, mutations were the most frequently found also in the residue *Q61*, 16/35 (45%) of all *KRAS* mutations: five in rrMM and nine in ndMM, two patients with unknown data.

Among 14 cases with *DIS3* mutations from patients with known clinical data, 9/14 were found in ndMM (64%) and 5/14 in rrMM (36%). Interestingly, isolated mutations in *DIS3* were only found in ndMM - 2/14 (14%).

BRAF mutations were found in 10 cases in total. Nine of 10 mutants *BRAF* with known data were almost equally distributed in ndMM 5/9 (56%) and 4/9 rrMM (44%). Six out of those nine were single mutations (67%), of which 5/6 cases were in ndMM and one in the rrMM group.

Among other mutations, those within *TRAF3* were more common in ndMM 6/9 (67%).

The two *IDH1* mutations were found only in ndMM, being probably of subclonal origin, considering their low VAF, Table 2.

3.3 | Primary outcome

We investigated the impact of different mutations on treatment outcomes in patients with ndMM receiving VRd in a cohort of 78 patients. In this group, 38 patients out of 78 (49%) had a mutation in MAPK pathway (*NRAS*, *KRAS* or *BRAF*). Remarkably, only 21 of these 38 patients (55%) achieved a CR; while patients with wild type of *NRAS*, *KRAS* or *BRAF* showed higher CR rate—34/40 or 85% (OR 0.22, 95%CI [confidence interval] 0.06149–0.7082, $p = 0.006$), as shown in Figure 3.

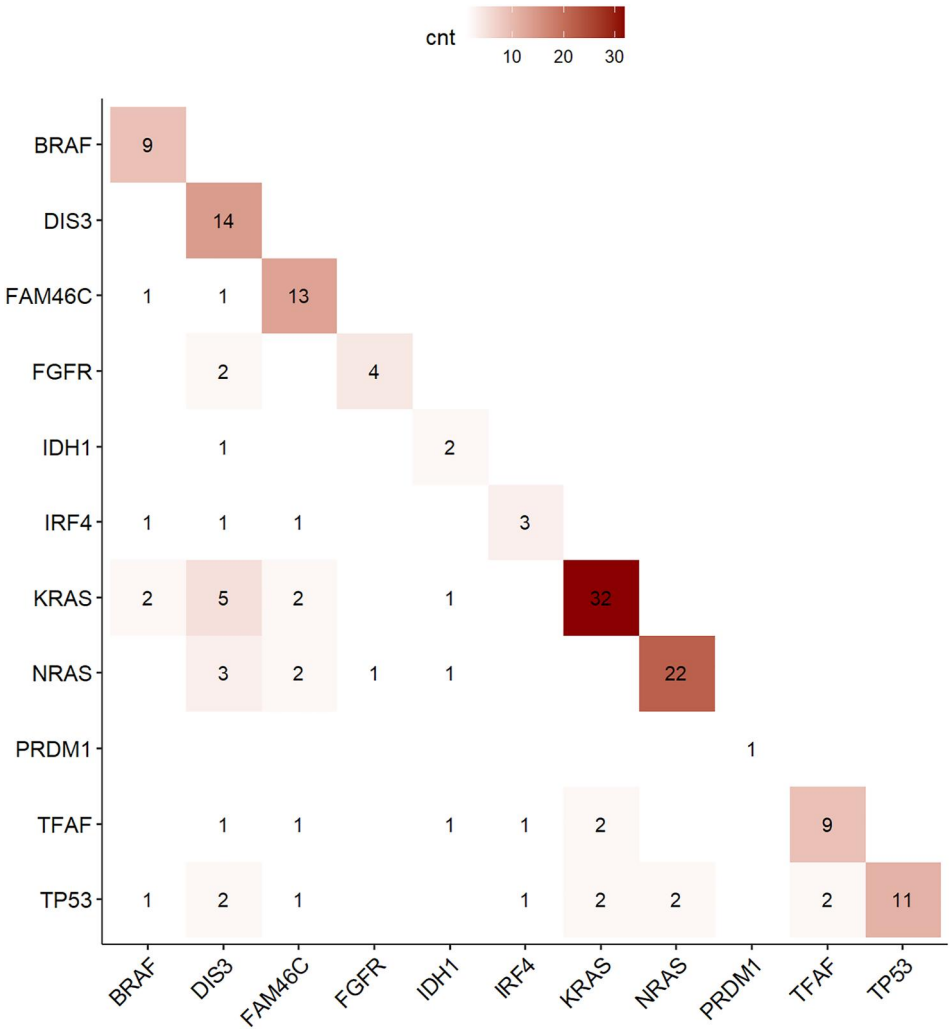

FIGURE 1 Number of mutations, their frequency and overlap in multiple myeloma (MM) samples.

Concerning effect of a specific mutation, *NRAS p.Q61K* mutant was associated with reduced probability to obtain CR in ndMM, as compared to other mutations (OR 0.0912, 95%CI 0.0018–0.9948, *p*-value 0.02).

None of the other analyzed mutations was found to significantly impact the CR rate in this study.

3.4 | Secondary outcome

As secondary outcome, we focus our attention on patients with ndMM, homogeneously treated by VRd. The correlation of mutational analysis with treatment outcome showed a negative influence of mutation in MAPK pathway –members *NRAS*, *KRAS* and *BRAF* on the probability to obtain CR in newly diagnosed myeloma as shown in Figure 3 (OR 0.22, $p = 0.006$). The negative influence of these mutations is also seen in the analysis of PFS, with a significantly shorter median PFS of 33.9 weeks compared to those without any of these mutations ($p < 0.0001$, Figure 3).

A similar negative impact of these mutations was found on OS. The presence of any mutation in *NRAS*, *KRAS* or *BRAF* genes was associated on average with a loss of 13.5 weeks of life (95%CI −26 to −1.04 weeks, $p = 0.03$).

4 | DISCUSSION

With the rapidly evolving methods of molecular analysis in the last decade, the use of NGS becomes more and more accessible for the routine diagnostic workup of patients with MM.[31]

The pattern of genomic lesions, found in our MM sample's cohort is in line with previously published NGS based studies, where the majority of alterations were detected in members of MAPK pathways (*KRAS*, *NRAS* and *BRAF*), *DIS3*, *FAM46C* and *TP53* genes.[3–5,13,14,36,37]

As expected, the comparison between mutational status of ndMM and rrMM showed a greater mutational load in rrMM versus ndMM and significantly higher frequency of *TP53* mutations. These results highlight again the importance of *TP53* disruption for MM relapse and progression MM.[35,38]

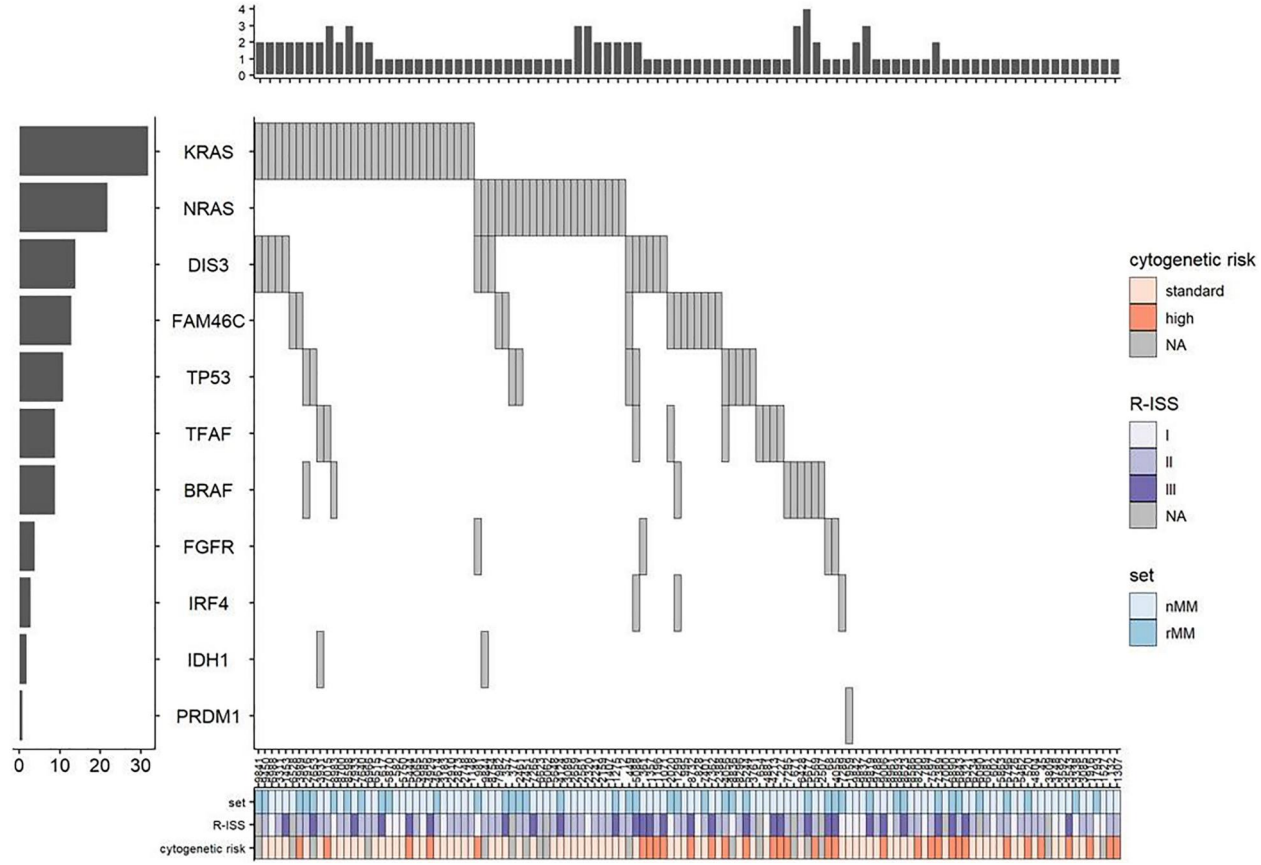

FIGURE 2 Types of mutations, sorted by frequencies and clinical data such as Revised International Staging System (R-ISS), cytogenetic risk and first diagnosis (newly diagnosed multiple myeloma (nMM)) versus relapse (relapsed/refractory multiple myeloma (rMM)) (set).

Concerning the correlation between VRd –outcome and genomic lesions in ndMM, mutations in *NRAS*, *KRAS* and *BRAF* genes were found to be associated with lower probability of obtaining CR. Moreover, as shown in the results, mutations in these MAPK-pathway's members seem to shorten PFS, Figure 3.

The oncogenic potential of MAPK-pathway's activation via mutation in *KRAS*, *NRAS* and *BRAF* has already been described in solid tumors, including melanoma, colorectal and pancreatic cancer[39–43]

In MM, prognostic and therapeutic significance of mutations in MAPK pathway members, as well as the timing of their appearance during disease progression have also been assessed.

KRAS, *NRAS* and *BRAF*-mutant are the most frequent in MM and are the first to appear in monoclonal gammopathy of unknown significance (MGUS).[5,36,44,32,33,37,45–47] The high frequency of involvement and early timing of appearance of MAPK pathway activation points to its essential function for malignant plasma cell clone survival and expansion.[36,48]

However, there seem to be some discrepancies regarding impact of outcome of mutations in *NRAS* and *KRAS*. While earlier works studying the influence of mutations in RAS –family members (*NRAS* and *KRAS*) on PFS and OS in MM, suggested a negative prognostic significance of *KRAS* mutations, a more recent study of a small cohort of patients suggested that rather mutant *NRAS*, but not *KRAS* could diminish the sensitivity to proteasome inhibitors (PI) based treatment in MM.[46,49,50] A Laganà1,2 et al., have additionally shown in a large integrative analysis in a waste MM cases series, that mutant *NRAS* could rather be consider as a favorable prognostic biomarker.[38] These studies present obviously several limitations, including low sample numbers mostly from single institutions, mixed analysis of both ndMM and rrMM patients and lack of longitudinal observation. Furthermore, patients were treated according to currently outdated chemotherapy regimens or received single agent treatment.[49–51]

A group from MD Anderson Cancer Center recently showed that activated MAPK signaling could enhance proteasome capacity in neoplastic plasma cells, thus inducing and hence a resistance to PI based treatment.[52] Based on the findings of Shirazi and co-authors, there could be a greater rationale for targeting MAPK-activated MM with *BRAF* or *MEK* inhibitors.

Another recent publication defined an activating interaction between mutated *KRAS* and *NRAS* and mammalian target of rapamycin (*MTOR*)-signaling in MM.[53] Therefore, the addition of *MTOR* inhibitors to PI-backbone regimens may be another possible option to overcome MAPK-driven resistance to standart triplets in MM.[52]

Concerning specific mutations and treatment outcome, *NRAS Q61* hot-spot mutations seems in our cohort to be associated with worse outcome in VRd treatment. *NRAS Q61* hot-spot involvement, mostly the *Q61*R, but also *Q61*K have already been described

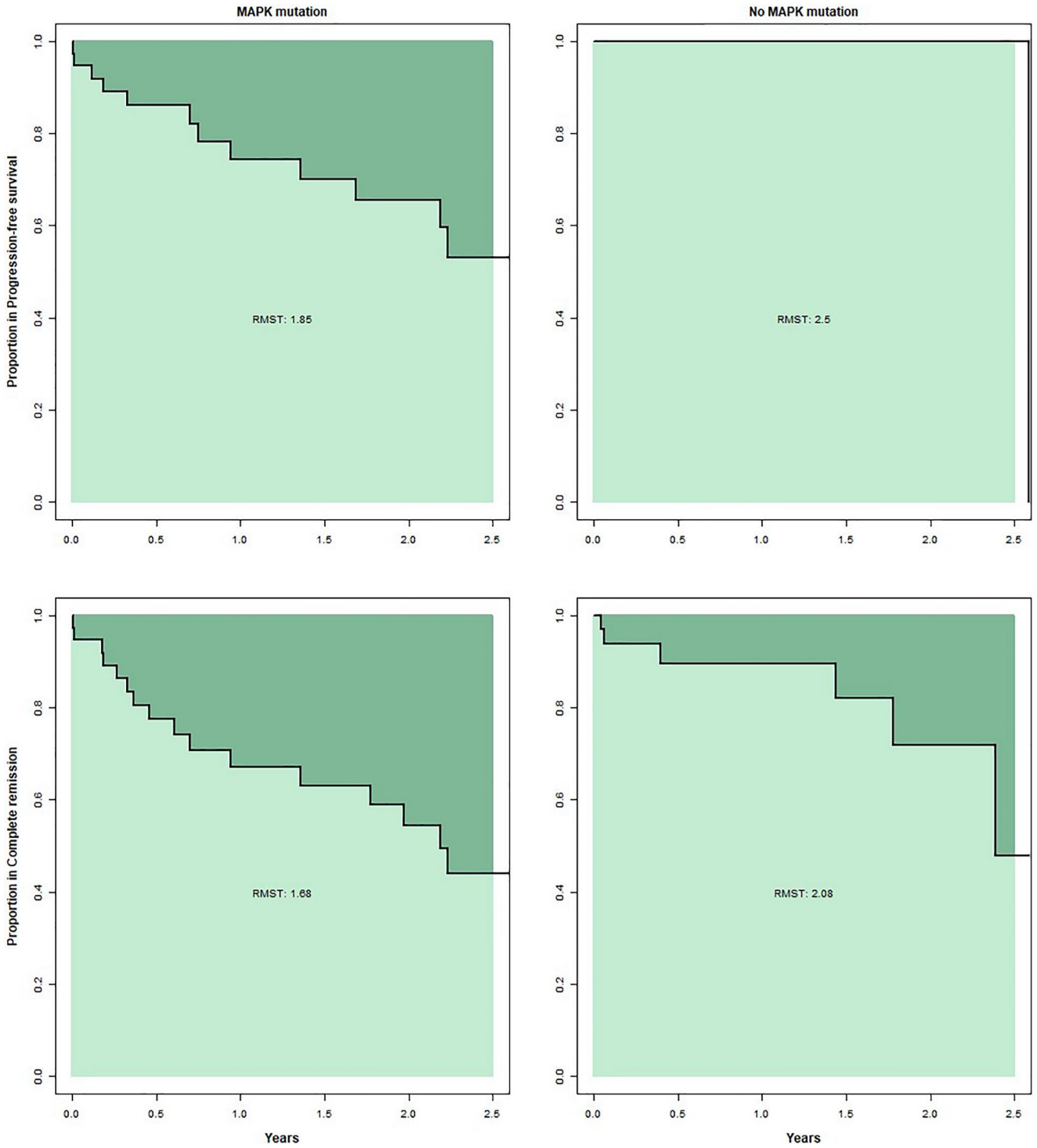

FIGURE 3 Influence of MAPK pathway activation via *NRAS*, *KRAS* and *BRAF* mutations on progression free survival (PFS) (upper curves) and treatment outcome (bottom part) in newly diagnosed multiple myeloma (nMM) patients, receiving PI-based regimens.

in different neoplasms, including other lymphoid malignancies, melanoma, central nervous system tumors and colon cancer in association with worst outcome, metastatic potential and treatment resistance.[54–65] Regarding its role in myeloma genesis, Wen et al. have recently shown that expression of *NRAS Q61*R mutant and *MYC* in germinal center B- cells in a VQ murine model, leads to higher proliferation of plasma cells in MM.[66]

Concerning a putative mechanism of tumor resistance induced by mutations in *Q61* in *NRAS*, it seems that mutations located in *Q61* codon could impair more severely RAS-intrinsic GTPase function, than affecting the *G12* codon.[67] In addition, an important interaction between *NRAS Q61* mutations and *p16INK4a* inactivation in *NRAS Q61*K transgenic mice has been shown.[68] Therefore, it seems that *Q61 NRAS*-mutant shows a stronger oncogenic activity.

In conclusion, activation of MAPK pathway via mutations in *NRAS*, *KRAS* and *BRAF* genes seems to have a negative impact on outcome in patients with ndMM treated by standard PI/IMiDs -based triplets, like VRd. Furthermore, *NRAS Q61K* -mutant appears to be associated with worst outcome in this setting. Our findings look especially relevant in context of increasing number of therapeutic MAPK-pathway inhibitors in development (*BRAF* and *MEK*- inhibitors) which could be added to PI/IMiDs-backbones. Larger prospective studies are needed to confirm our results.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare that are relevant to the content of this article. Author Camille Perroud declares that she has no conflict of interest. Author Dario Thurian declares that he has no conflict of interest. Author Martin Andres declares that he has no conflict of interest. Author Martin Künzi declares that he has no conflict of interest. Author Gertrud Wiedemann declares that she has no conflict of interest. Author Sacha Zeerleder declares that he has no conflict of interest. Author Ulrike Bacher declares that she has no conflict of interest. Author Thomas Pabst declares that he has no conflict of interest. Author Yara Banz declares that she has no conflict of interest. Author Naomi A. Porret declares that she has no conflict of interest. Author Ekaterina Rebmann declares that she has no conflict of interest.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to institution-related patient identity restrictions.

ETHICS STATEMENT

All procedures were in accordance with the ethical standards of the respective local research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed written consent was obtained from all individual participants included in the study. The approval for access to the clinical and personal patient data used in the study was granted by the Cantonal Ethics Committee of Bern, Switzerland (Kantonale Ethikkommission Bern). The study was approved by the Cantonal Ethic Commission Bern, Switzerland (Kantonale Ethikkommission Bern); Decision number: 2022-00097.

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PEER REVIEW

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