Unique molecular assay (UMA): a next-generation sequencing targeted panel for efficient and comprehensive genomic profiling and risk stratification of multiple myeloma

Andrea Poletti,[1,2*] Barbara Taurisano,[1,2*] Gaia Mazzocchetti,[3,4] Marta Lionetti,[5] Marina Martello,[1,2] Viola Meixian Vuong,[1,2] Vincenza Solli,[1,2] Giulia Marzocchi,[1,2] Akihiro Maeda,[6] Ilaria Vigliotta,[1] Enrica Borsi,[1] Silvia Armuzzi,[1,2] Ignazia Pistis,[1] Alessio Marella,[5] Sonia Fabris,[6] Paola Tacchetti,[1] Katia Mancuso,[1,2] Ilaria Rizzello,[1,2] Lucia Pantani,[1] Nicoletta Testoni,[1,2] Michele Cavo,[2] Elena Zamagni,[1,2] Niccolò Bolli[5,6] and Carolina Terragna[1]

[1]IRCCS Azienda Ospedaliero-Universitaria di Bologna, Istituto di Ematologia "Seràgnoli", Bologna; [2]Dipartimento di Scienze Mediche e Chirurgiche, Università di Bologna, Bologna; [3]FABIT-Department of Pharmacy and Biotechnology, University of Bologna, Bologna; [4]Computational and Chemical Biology, Italian Institute of Technology (IIT), CMP3VdA, Aosta; [5]Department of Oncology and Hemato-Oncology, University of Milan, Milan and [6]Hematology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

AP and BT contributed equally as first authors..

Correspondence: N. Bolli
niccolo.bolli@unimi.it

C. Terragna
carolina.terragna@unibo.it

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Abstract

Multiple myeloma (MM) is characterized by genetic abnormalities in plasma cells, requiring precise genomic characterization for effective risk stratification and treatment. This study presents the Unique Molecular Assay (UMA) panel, a targeted DNA-sequencing approach designed to capture critical genomic aberrations in MM, including canonical immunoglobulin heavy chain translocations (t-IgH), copy number alterations (CNA), and mutations in 82 genes. The UMA panel is the first MM sequencing panel validated against traditional methods like fluorescence *in situ* hybridization (FISH) and SNP arrays across two laboratories for clinical-grade accuracy and reproducibility. The study included 150 patients whose DNA samples were analyzed using the UMA panel, achieving a median coverage of 233X with a requirement of ≥4 million reads per sample. The UMA panel demonstrated high concordance with FISH in detecting both CNA and t-IgH, achieving a balanced accuracy of over 93%. Moreover, inter-laboratory validation confirmed the robustness and reliability of the panel on genomic alteration calls. Importantly, the UMA panel enabled precise risk stratification based on the Second Revision of the International Staging System (R2-ISS), identifying high-risk features such as *TP53* mutations and genome-wide CNA. This comprehensive and cost-effective genomic profiling tool supports clinical decision-making and personalized treatment strategies in MM. The validated performance and scalability of the UMA panel suggest its potential to complement traditional diagnostic methods, offering detailed insights into the genomic landscape of MM.

Introduction

Multiple myeloma (MM) is a neoplasm arising from genetic abnormalities acquired in the germinal center at the expense of a mature B lymphocyte. These include translocations of recurrent oncogenes in the locus of the immunoglobulin heavy chain (t-IgH) or recurrent trisomies of multiple chromosomes (chr), called hyperdiploidy; these two, mostly mutually exclusive events, are considered as initiating events. Clonal cells go on to migrate in the bone marrow (BM) and differentiate into plasma cells (PC). Many functional properties of normal PC, including antibody secretion, are not lost upon transformation. Consequently, the hallmarks of MM are an excess of PC in the BM and the presence of a monoclonal protein (MP) in the serum.[1,2] One major issue at MM diagnosis is whether the response to treatment and/or survival of the patient can be predicted, and by which factors. Recently, most of the risk staging

systems in use adopted the presence of genetic abnormalities identified by fluorescence *in situ* hybridization (FISH) in CD138-purified PC as additional factors contributing to prognosis. In particular, the Revised International Staging System (R-ISS) mandates that t(4;14), t(14;16), del(17p) are analyzed as poor prognostic factors,[3] while in the Second Revision of the ISS (R2-ISS), the t(14;16) is dropped in favor of the presence of gain/amplification of chr(1q).[4]

The genomic characterization of MM by means of next-generation sequencing (NGS)[5-8] has greatly expanded the catalog of genetic drivers of the disease and suggested novel prognostic markers.[9-13] In addition, novel targeted immunotherapies with unprecedented potential are entering the market, and analysis of refractory cases suggest for the first time in MM that target gene mutations and/or deletions are predictive of response to treatment.[14-17] Therefore, a deeper genomic characterization of MM at diagnosis, by means of NGS, is more suitable for analyses of several genetic regions and gene sequences than traditional approaches (e.g., FISH).[18] In the past, several clinical-grade approaches have been proposed, all based on targeted DNA-sequencing. The main differences consisted in the target region chosen and in the analytical method employed. Many were based on pulldown of the whole IgH region to detect an IgH translocation and of thousands of SNP evenly spaced across the genome to allow detection of abnormalities in focal regions and copy-neutral loss of heterozygosity.[19-21] Others offer a more comprehensive characterization of MM translocations, particularly those involving MYC and the IgH loci,[22] at the expense of a larger target region, increasing complexity and cost of the analysis. However, no such panel has been tested across laboratories for reproducibility against the gold standard, represented by FISH, or against other genomic technologies, such as SNP arrays and/or ultra-low pass whole genome sequencing (ULP-WGS).

In an effort to reduce the target region footprint, validate the reproducibility of data when sequencing is performed in different laboratories, and propose a streamlined analysis that could be applied for clinical-grade diagnostics, here we present a Unique Molecular Assay (UMA), a customized target enrichment panel that captures gene mutations, CNA, and t-IgH in MM.

Methods

Patient cohort

This study enrolled 130 newly diagnosed multiple myeloma (NDMM) patients, 20 smoldering MM (SMM) patients, and 13 healthy donors, all with existing cytogenetic or SNP array data. Participants were recruited in Bologna (IRCCS Azienda Ospedaliero-Universitaria di Bologna Policlinico Sant'Orsola, Seràgnoli Institute of Hematology) and Milan (Section of Hematology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico), Italy. All provided written informed consent and received standard clinical treatment. The study complied with the Declaration of Helsinki and was approved by the local ethics committees in Bologna (Protocol 167/2019/Sper/AOUBo) and Milan (Provision N. 575 of 29/03/2018).

A total of 207 DNA samples from BM-CD138$^+$ cells (*Online Supplementary Methods*) were sequenced using the UMA panel (Table 1). The Bologna (BO) cohort (136 samples from 130 NDMM patients) was mainly used for intra-laboratory validations; 30 of these patients were also part of the inter-laboratory validation (BO-Milan [MI] cohort), which included also 20 SMM samples (MI-BO cohort) that were delivered from Milan to Bologna. Two technical cohorts were also analyzed: the Panel of Normal (13 healthy donor DNA) for focal copy number calibration, and the Dilution Test (8 diluted DNA with a known *BRAF* mutation) for determining the variant allele frequency detection limit.

Customized capture next-generation sequencing panel – the Unique Molecular Assay panel

A multiplex customized capture NGS assay, developed in-house and named the UMA panel, was designed to detect the most common and relevant genomic aberrations described in MM patients.[5-13] It includes the most frequent t-IgH, whole genome CNA and single nucleotide variants (SNV) in 82 genes, for a total footprint of 460.4 Kbp. The panel was designed using the SureSelect Agilent Design System (Agilent Technologies, Santa Clara, CA, USA). Details on the strategy applied to design the panel are reported below. The library preparation of the UMA panel is described in the *Online Supplementary Appendix*. Fastq files were then analyzed by using a customized bioinformatic pipeline (see *Online Supplementary Methods*).

Fluorescene *in situ* hybridization and single-nucleotide polymorphism arrays

Fluorescence *in situ* hybridization panels included probes for t(4;14)(p16;q32), t(6;14)(p21;q32), t(11;14)(q11;q32), t(14;16)(q32;q23), and t(14;20)(q32;q12) from CytoCell® FISH probes (Oxford Gene Technology, Cambridge, UK). All FISH testing was performed in the Bologna and Milan clinical cytogenetics laboratories by analyzing between 100 and 500 cells per sample. FISH assays were performed only on those patients having sufficient CD138$^+$ cells.

Single-nucleotide polymorphism arrays (Cytoscan-HD, Thermo Fisher Scientific, Waltham, MA, USA) were performed following the manufacturer's protocol with 2.67 million probes. Data quality analysis was performed using Affymetrix ChAS 4.2 software. For each patient, the CNAs profile was obtained by Rawcopy R package (v1.1).

Milan next-generation sequencing customized panel data generation and processing

Some samples had been previously sequenced in Milan with independent approaches and were used for validation of

Table 1. Description and purpose of use of all the five cohorts of samples included in the study.

Cohort	Cohort and study center ID	N of samples	Patients, N	Aim	Description
Main analysis cohort	BO	136	130 NDMM	Intra-lab validation and performance evaluation with FISH and SNP arrays calls.	Intra-lab (BO) validation of wetlab processing and data analysis pipeline.
Validation cohort 1	BO-MI	30	30 NDMM (overlapping with BO)	Inter-lab wetlab processing validation.	30 DNA samples analyzed in BO with UMA panel have been sent to MI to be processed and sequenced in an external laboratory with UMA panel. Generated fastq files were sent back to BO and analyzed by BO pipeline to check for concordances.
Validation cohort 2	MI-BO	20	20 SMM	Inter-lab pipeline validation.	20 DNA samples, already analyzed by MI wetlab and pipeline, have been sent to BO to be processed and sequenced also by BO (UMA panel) and checked for concordances with MI calls.
Normal samples	PON	13	-	Creation of a PON.	PON is required for on-target CNA pipeline. It consists of 13 samples with normal (diploid) CN profile from buccal swabs.
Dilution tests	dil-test	8	-	Experimental validation of the VAF limit of detection for mutations calls.	Four dilution levels of a DNA sample containing a known mutation of *BRAF* gene were prepared in twin replicates, for a total of 8 samples.
Total	-	207	150	-	-

BO: Bologna; CNA: copy number alterations; N: number; NDMM: newly diagnosed multiple myeloma; FISH: fluorescence *in situ* hybridization; SNP: single nucleotide polymorphism; PON: Panel of Normal; MI: Milan; SMM: smoldering multiple myeloma; UMA: unique molecular assay; VAF: variant allele frequency.

the UMA panel, as described in the *Online Supplementary Methods*. Bioinformatic data analysis procedures are also detailed in the *Online Supplementary Methods*.

Results

The Unique Molecular Assay panel design: the next-generation sequencing strategy

The UMA was designed to meet two critical clinical requirements: to obtain a detailed genomic profile of MM patients for prognostic risk assessment, and to balance costs and benefits of NGS for routine clinical use. This novel NGS assay was developed to provide comprehensive genomic information for clinicians while minimizing panel size (total panel size = 0.46 Mbp) (*Online Supplementary Table S3*), sequencing costs, data storage, processing time and facilitating interpretation of results by means of an *ad hoc* bioinformatic pipeline.

The UMA panel targets three main types of MM genomic alterations: CNA, t-IgH and gene mutations (SNV and indels). The overall NGS panel design strategy is detailed below.

• CNA: the UMA panel targets whole genome CNA by leveraging off-target DNA captured during hybridization, which is sequenced alongside on-target regions. This approach provides shallow whole genome coverage suitable for detecting broad CNA, such as chromosome arm alterations.[23,24] By combining the off-target approach with a customized on-target CNA calling method, we developed a strategy which leverages all available sequencing reads to call both broad and focal CNA. This strategy eliminates the need for thousands of SNP spaced across the genome, commonly employed in other panels to call broad CNA, while ensuring high resolution for detecting both whole genome-level and gene-level CNA in regions of interest.

• t-IgH: in MM pathogenesis, t-IgH arises from class switch recombination events, leading to breakpoints that frequently cluster in specific regions of the IgH locus.[25] To capture these, we focused on known t-IgH breakpoint positions derived from large WGS studies (N=583 and N=176 t-IgH from CoMMpass [IA20][26] and an internal dataset, respectively; total N=757 t-IgH). Once the t-IgH breakpoints distribution had been defined (*Online Supplementary Figure S1*), we chose to target each unique IgH region where breakpoints had been detected. This resulted in a total of 170 regions with an overall footprint of 92.9 kbp. Given that the total IgH locus spans 1235.3 kbp, this approach enabled a 92.5% reduction in the panel's target IgH design.

• Gene mutations: 82 genes (detailed in *Online Supplementary Tables S1, S2)* were selected as target, according to their relevance to MM, as being: 1) either oncogenes or tumor suppressors reported in the COSMIC databases

and MM driver genes in large genomic studies;[10,27-32] 2) part of frequently altered pathways in MM (e.g., *NFKB*, *MAPK*, cell cycle, DNA repair[2]); 3) known targets of MM therapies, involved in resistance mechanisms; 4) targets of focal amplifications or deletions, as defined by GISTIC2 analysis.[13]
To highlight the UMA panel's main advantages, the present design was compared to other previously reported MM panels employing different approaches (Table 2). This comparison highlighted significant reductions both in sequencing resources per patient and in resources needed for bioinformatic analysis and data storage (sequenced Mbp per sample), still maintaining the ability to detect pivotal MM genomic alterations (Figure 1). The UMA panel was thus seen to be a cost-effective and powerful tool for routine clinical use.

The Unique Molecular Assay panel implementation

Overall, a total of 146.1 Gbp were sequenced, with a median on target coverage of 233X (range: 59-414X) and a median of 4.8 million (M) total reads per sample (range: 1.4-14.2 M), including a median of 54.6% off-target reads (range: 36-81.4%). We performed experiments to test the optimal sequencing parameters to be able to call all the alterations confidently. We estimated that a median of 4 M reads/sample and a 210X on-target coverage was enough to call both t-IgH and mutations ≥5% VAF. This estimation is based on having a minimum of 5 variant supporting reads and achieving a detection probability of over 95%, according to binomial probability calculation.[33] In addition, our estimation permits us to obtain sufficient off-target reads to call genome wide chromosomal arm CNA (≥1.5M off-target reads, considering a range of 40-60% off-target percentage).
The NGS results, as obtained from the BO cohort (N=130), are depicted in Figure 2. The CNA were called both from the off-target and from the on-target reads signals, after CN signal correction and harmonization.[34] The most common broad CNA gains were on chr 9 (N=63) and 19 (N=62), while the most common losses were on chr 13 (N=55) and 14 (N=38). The most common focal CNA gains involved *CKS1B* (located on chr 1q) (N=26), while the most common losses involved *RB1* (chr 13q) (N=58). The pipeline for t-IgH calls allowed the identification of t(4;14) (N=26), t(11;14) (N=17), t(14;16) (N=6), t(14;20) (N=5), and t(6;14) (N=3) (Figure 2). We found a median on-target coverage of 233X (range: 17-400X) for gene mutations, ensuring reliable mutational calls. Considering variant allele frequency (VAF) ≥5% and selecting pathogenic variants (details in *Online Supplementary Methods*), we detected a total of 386 mutations (313 missense, 35 stopgain, 25 frameshift indels, 10 in-frame indels, 5 startloss, 2 stoploss) and one splicing mutation, with the most mutated genes being: *NRAS* (N=26), *KRAS* (N=22), *BRAF* (N=18), *ATM* (N=15), which are commonly reported as the most often mutated genes in MM (Figure 2).

Intra-laboratory validation

The intra-laboratory validation was performed in Bologna, on the BO cohort's samples (see Methods and *Online Supplementary Methods*). The aim of the intra-laboratory validation was to evaluate the overall performance of the UMA panel and compare its results with those obtained by gold-standard approach (i.e., FISH), with the purpose to identify, quantify and explain both concordances and discordances, thereby ensuring reliable and confident use of the UMA panel for defining patients' genomic profiles.

CNA analyses: UMA versus FISH

According to the quality of the samples, two dynamic thresholds, ranging from 20% to 40% clonality, were defined to detect focal and broad CNA and combined to call CNA (*Online Supplementary Methods* and *Online Supplementary Figure S2*). To confirm the CNA profiles, as described according to the UMA panel's calls, we compared the NGS results with those available from the baseline patients' clinical characterization, performed in daily practice. To this aim, CNA on chr 1q (CKS1B), 1p (CDKN2C), 13q (RB1), and 17p (TP53), as detected by FISH, were considered the benchmark for data comparison.
The comparison between the UMA and FISH was performed only for those patients having both evaluations. In particular, 9/121 (7.4%) del(17p), 38/81 (46.9%) del(13q), 34/109 (31.2%) amp(1q), and 9/96 (9.4%) del(1p) were detected by FISH (*Online Supplementary Figure S3A-D*). Notably, total numbers of patients analyzed may differ, as not all four FISH evaluations were available for all patients included in the BO cohort.
Overall, the comparison showed a Balanced Accuracy (BA) of 93.1%, with 312 CNA concordant negative, 79 concordant positive, 11 CNA identified just by FISH, and 5 just by the UMA panel, resulting in a Positive Predictive Value (PPV) of 94.0% and a Negative Predictive Value (NPV) of 96.6%. The NGS CNA detection sensitivity and specificity were 87.8% and 98.4%, respectively (Figure 3A).
All discordances (N=16) were manually reviewed, and the main reasons identified were: 1) borderline cut-offs (N=5) with sub-clonal FISH (10-30% clonality range) and negative UMA calls, close to the limit of detection; 2) possible Whole Genome Doubling (WGD) events[35] (N=6), leading to a homogenous ploidy shift, undetectable by NGS bulk analysis methods.[36] WGD was suspected in cases where the UMA and SNP array results were concordant and both discordant with FISH;[36] 3) focal CNA noisy signal (N=2) probably due to the still limited number of cases (13 samples) included in the PON and used for CN signal normalization and correction. After the review process, 3 discordant cases (0.73%) remained unexplained

t-IgH analyses: UMA versus FISH

The BO cohort was used to compare t-IgH calls as assessed by the UMA panel with FISH data of five common t-IgH

Table 2. Overview and comparison of all published multiple myeloma targeted sequencing panels.

Study	Kortüm *et al.*, 2014 (M3P panel)[45]	Jimenez *et al.*, 2016[46]	Bolli *et al.*, 2016[19]	Kortum *et al.*, 2016 (M3Pv2.0 panel)[47]	Yellapantula *et al.*, 2019 (myTYPE panel)[21]	Cutler *et al.*, 2021 (DMG 26 panel)[48]	Sudha *et al.*, 2022 (Myeloma Genome Project panel)[22]	Current paper (UMA panel)
Patients	50 MM samples	48 pts	Cell lines	142 pts	154 pts	76 pts, 4 cell lines	233 MM samples	209 samples (150 MM/SMM pts)
Detected alteration types	Mutations	IgH translocations, VDJ rearrangements, mutations	IgH translocations, VDJ rearrangements, mutations and CNA	Mutations, focal CNA	IgH rearrangements, CNA, mutations	Mutations	IgH translocations, MYC-translocations, CNA, mutations.	IgH translocations, CNA, mutations
Genes	47 genes	IGH + 6 genes: *NRAS*, *KRAS*, *HRAS*, *TP53*, *MYC*, *BRAF*	IGH + IGH partners + 182 genes + 2,538 SNP	77 genes	120 genes	26 genes	228 genes	IGH hotspots + 82 genes
Library prep	Amplicon-based	Capture-based	Capture-based	Amplicon-based	Capture-based	Capture-based or amplicon-based	Capture-based	Capture-based
Target dimension	2,875 amplicons 268 Kbp (estimated)	208 Kbp	2,992 Kbp	Not declared (estimated 154 Kbp)	2,060 Kb	99 Kbp (Capture); 117 Kbp (Amplicon)	4,320 Kbp (translocations) + 1,190 Kbp (CNA and mutations)	460 Kbp
Starting material	20 ng DNA	500 ng DNA	500 ng DNA	20 ng gDNA	100-200 ng gDNA	Not declared	100 ng gDNA	100 ng
Sequencer	Semiconductor technology (PGM, Life Technologies)	GS 454 FLX+ (Roche)	HiSeq 2000 (Illumina)	Ion Torrent Platform (Thermo Fisher)	HiSeq 2500 (Illumina)	Not declared	Nextseq (Illumina)	Miseq; NextSeq (Illumina)
Mean read length	400 bp	350 bp	75 bp	200 bp	101 bp	Not declared	75 bp	200 bp
Mean coverage	298 X (tumor); 221 X (germline)	>23,000 X	155 X	658 X (tumor); 492 X (germline)	651 X	1,081X (Capture); 1,026X (Amplicon)	150 X translocations + 450 X mutations	233 X
Total sequenced Mbp per sample (target dimension x mean coverage)	80 Mbp / sample	4,784 Mbp / sample	463 Mbp / sample	101 Mbp / sample	1,341 Mbp / sample	107 Mbp / sample (Capture); 120 Mbp /sample (Amplicon)	1,183 Mbp / sample	107 Mbp / sample

CNA: copy number aberrations; gDNA: genomic DNA; MM: multiple myeloma; prep.: preparation; pts: patients; SMM: smoldering MM; SNP: single nucleotide polymorphism.

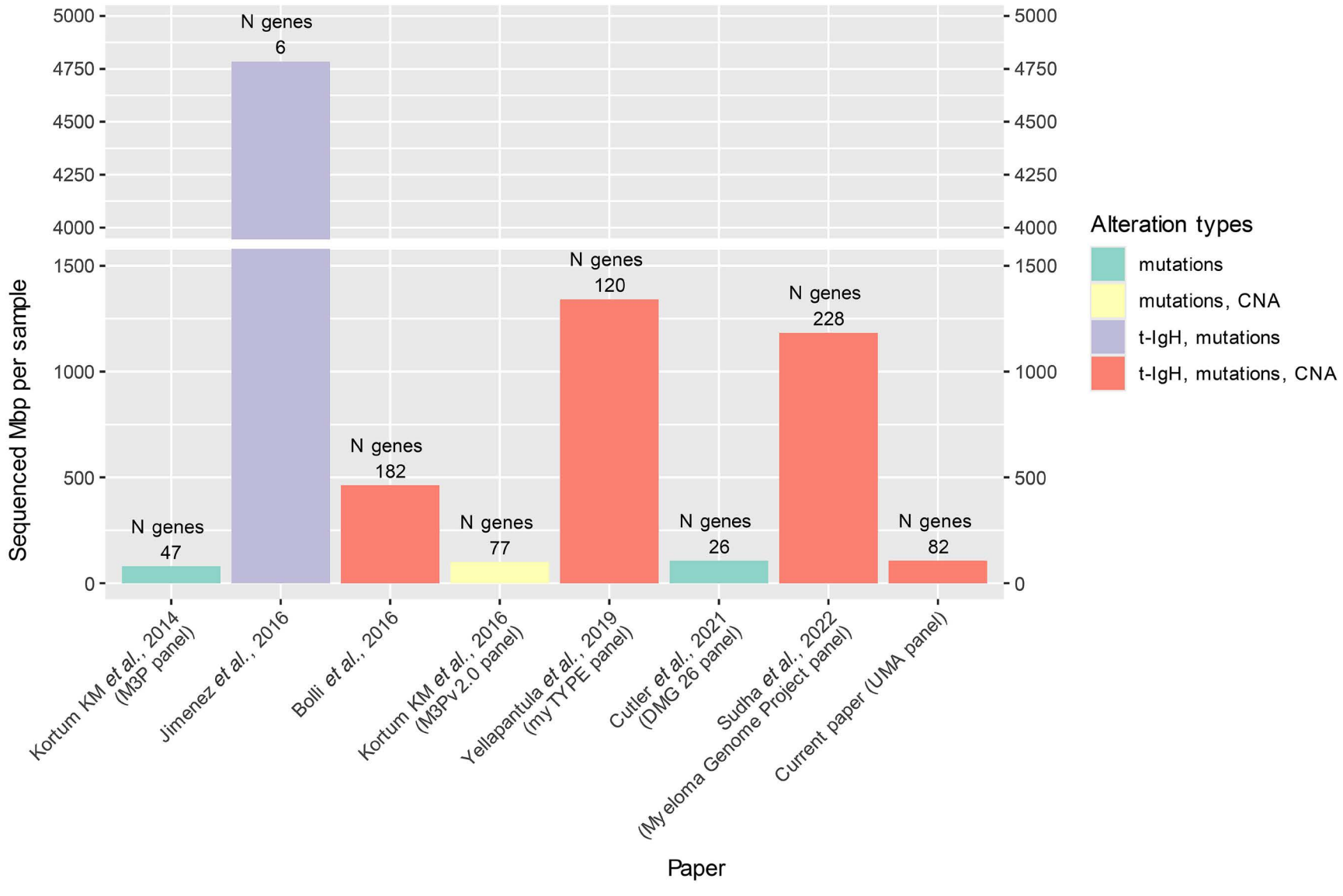

Figure 1. Efficiency of multiple myeloma targeted next-generation sequencing panels in our literature review. All the considered papers are represented along the X axis, while the Y axis quantifies the sequenced Mbp per sample (target dimension multiplied by target coverage). The detected alteration types of panels are color coded. It is noticeable that the Unique Molecular Assay (UMA) panel stands out for its efficiency among panels which detect the complete set of alteration types (translocation that involves IGH gene [t-IgH], mutations, and copy number alterations [CNA]). MM: multiple myeloma; NGS: next-generation sequencing.

routinely tested by FISH, including t(4;14), t(11;14), t(14;16), t(6;14), and t(14;20). Again, this comparison was performed only for patients having both the evaluations. A total of 44 positive FISH t-IgH calls were available for this analysis, in detail: 17/81 t(11;14) (21.0%), 18/120 t(4;14) (15.0%), 2/80 t(14;20) (2.5%), 4/103 t(14;16) (3.9%), and 3/77 t(6;14) (3.9%) were observed by FISH (*Online Supplementary Figure S3E-I*). The total numbers of patients analyzed may differ for the reasons explained above.

Overall, the comparison showed a BA of 94.1%, with 415 t-IgH concordant negative, 39 concordant positive, 5 t-IgH identified just by FISH, and 2 just by the UMA panel, resulting in a PPV of 95.1% and a NPV of 98.8%. The sensitivity and the specificity of the UMA assay to detect t-IgH were 88.6% and 99.5%, respectively (Figure 3B). All discordant cases were manually reviewed and discordances were attributed to: 1) non-canonical breakpoints on partner gene, mapping in the proximity (but outside) of the FISH probes' hybridization region (N=2), with UMA positive and FISH negative results; 2) multiple t-IgH (N=1), with the UMA panel detecting two t-IgH in the same patient (1 clonal and 1 subclonal) and FISH detecting just one. After the review process, 4 discordant cases remained unexplained (N=4, 0.86%).

Inter-laboratory validation

The inter-laboratory experiments were designed to validate both the wet-laboratory procedures and the bioinformatic pipeline. These experiments involved a subgroup of 50 samples collected from 50 patients: 30 samples (BO-MI cohort) were analyzed in Bologna with the UMA panel, with the wet-lab procedures then repeated in Milan on the same samples. Additionally, for 20 samples (MI-BO cohort), the UMA panel results from Milan were compared with data previously generated in Milan using alternative methods. This approach allowed for a thorough comparison of results obtained from different methods on the same samples.

BO-MI genomic call validation

The BO-MI cohort includes samples that have been sequenced both in Bologna and in Milan. Resulting CNA and t-IgH calls were also compared with previously available FISH and SNP array data, to further validate results. SNV

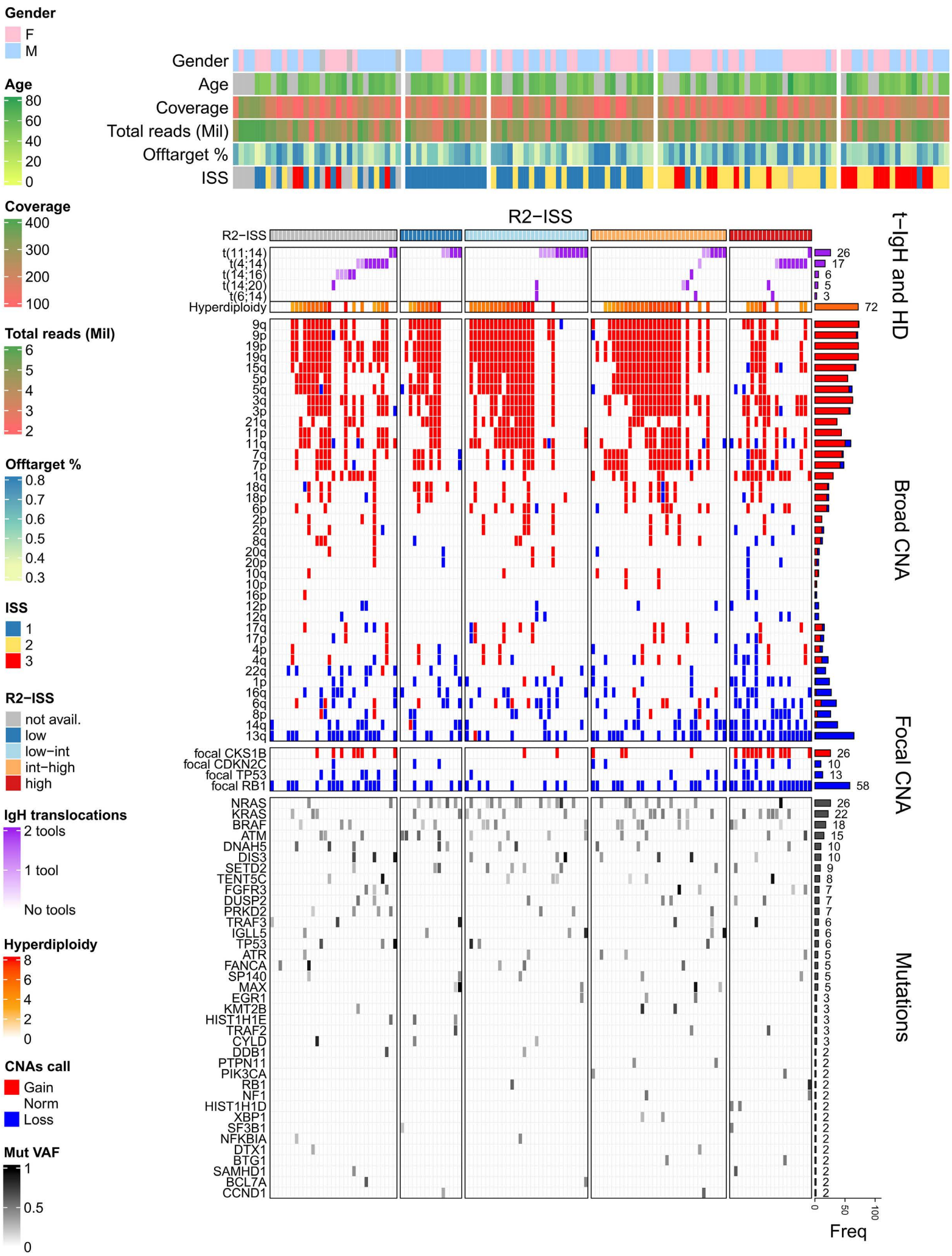

Figure 2. Unique Molecular Assay panel summary heatmap. Columns represent newly diagnosed multiple myeloma (NDMM) patients included in the main cohort (Bologna [BO], N=130), while rows represent all detected genomic alterations by the Unique Molecular Assay (UMA) panel. To avoid excessive matrix size, only mutations with count >1 are showed. Columns' heatmap sections correspond to the Second Revision of the International Staging System (R2-ISS) patients' class, while rows' heatmap sections correspond to the different alteration types detectable with the UMA panel (translocation that involves IGH gene [t-IgH], hyperdiploidy [HD], broad and focal copy number alterations [CNA], and gene mutations). avail.: available; F: Female; freq.: frequency; M: Male; Mil: millions; VAF: variant allele frequency.

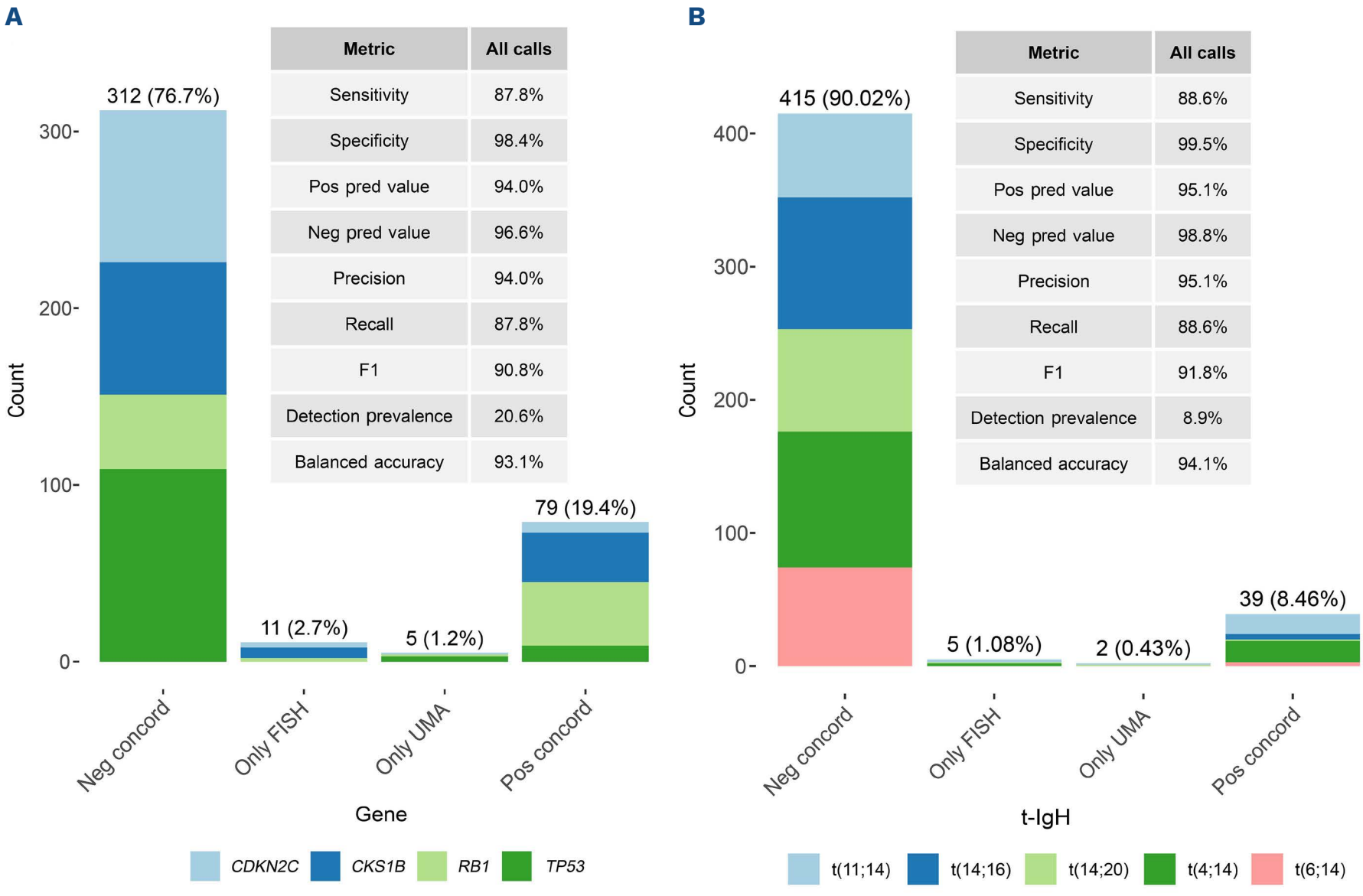

Figure 3. Intra-laboratory validation of the Unique Molecular Assay panel's calls in the Bologna cohort compared with gold-standard fluorescence *in situ* hybridization calls. The Bologna cohort, N=129 patients. (A) Stacked histogram which summarizes the overall number of concordant and discordant copy number alteration (CNA) calls between the Unique Molecular Assay (UMA) panel and fluorescence *in situ* hybridization (FISH), over the four considered target genes. In the nested table, all the relevant performance metrics are reported (using FISH data as reference). (B) Stacked histogram which summarizes the overall number of concordant and discordant translocations (t) that involve IGH gene (t-IgH) calls between UMA and FISH, over the five considered t-IgH. In the nested table, all the relevant performance metrics are reported (using FISH data as reference). Neg Concord: negative concordance; Pos Concord: positive concordance.

calls were compared between the two laboratories. Notably, even though the resulting sequencing metrics (i.e., total reads, on- and off-target percentages, target coverage) were not completely homogeneous between the two centers (*Online Supplementary Table S4*), overall, the genomic calls almost overlapped, highlighting the robustness of the UMA panel under different experimental conditions (Figure 4).

In detail, a high number of CNA (clonality cut-off 40%) were called from both laboratories (354 amplifications, 86 deletions), with a concordance of 93.02%. The two replicate profiles' overlap was also explored at segment level, with a more stringent cut-off (20% clonality), obtaining an average concordance of 88% (details in *Online Supplementary Methods*). This pairwise matching analysis allowed us to measure the concordance in terms of CN between common segment analyzed in replicates, considering the different states of clonality. This resulted in an overall good match between replicates coming from both laboratories and the SNP array profile (MI *vs*. SNP array mean concordance: 94.5%; BO *vs*. SNP array mean concordance: 94.4%) (Figure 4A).

Overall, 99% of t-IgH were called from both laboratories (Figure 4B). The concordance with FISH showed 100% agreement with both positive (N=11) and negative (N=78) panel calls.

Finally, 91.8% of SNV (112/122 with VAF ≥5%) were called from both laboratories (Figure 4C-E), with most variants showing a similar VAF in their replicates (Pearson test, R=0.86, *P*<0.001). These results were achieved by excluding sequencing errors (see *Online Supplementary Methods*); remaining discordances (highlighted for 10/122 variants) could be mainly attributed to: 1) low coverage (N=6), which prevents a correct SNV detection, causing a call discrepancy; 2) sub-clonality (N=2); 3) variant misclassification by one of the SNV calling tools filter (Mutect2 multiallelic or haplotype) (N=2). These pitfalls might be overcome by im-

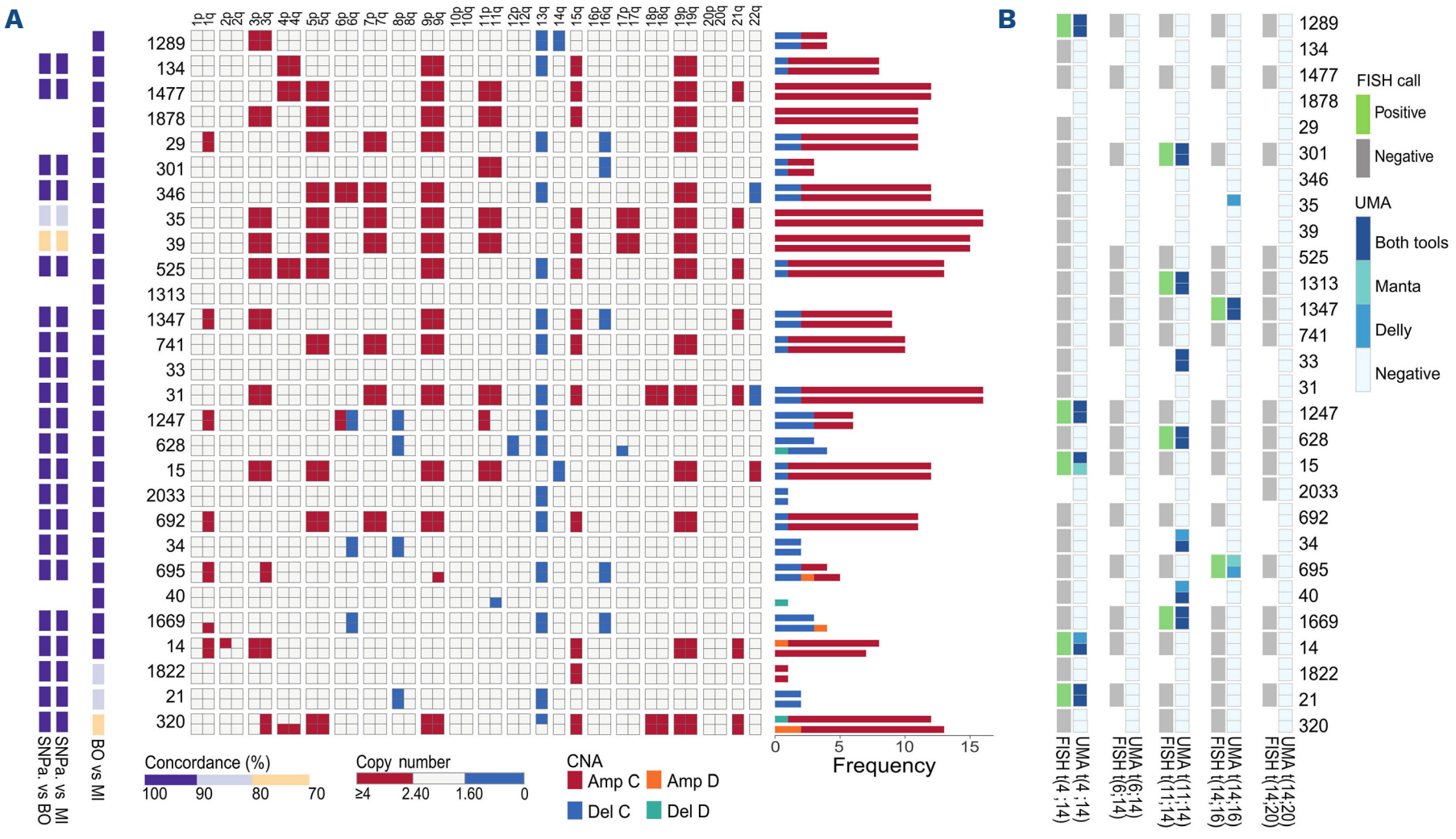

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Figure 4. Bologna-Milano interlab validation results. (A) The plot displays the copy number (CN) profiles of the Bologna-Milano (BO-MI) cohort DNA, overlaying the BO sample (top line) on the MI replicate (bottom line). Red indicates amplifications above 40% clonality, while blue shows deletions below 40%. (Right) Frequencies of events in each profile are shown; amplifications and deletions found in the replicate are colored red and blue, respectively, with discordances marked in orange and petrol blue. (Left) Concordance rate of the pairwise matching analysis at the segment level (BO-MI CN matching) is presented. Additionally, the samples were compared using single-nucleotide polymorphism array (SNParray) (SNParray concordance – MI and SNParray concordance – BO). Concordance percentages are displayed on a scale from purple to yellow. (B) Translocations (t) involving the IGH-region (t-IgH) were evaluated: t(4;14), t(11;14), t(14;16), t(6;14), and t(14;20), and compared with fluorescence *in situ* hybridization (FISH) results. Again in correspondence to the sample code, the top line represents the BO sample and the bottom line represents the MI replicate. For t(4;14) and t(11;14), replicates showed 100% concordance, with 5 and 7 patients testing positive, respectively. Three patients were positive for the t(14;16) event, although this translocation was not detected in the external replicate DNA 35. No translocations were detected for t(6;14) and t(14;20), all consistent with negative FISH results. The UMA panel calls were colored according to the number of tools used, on a scale of blue (negative, only Manta, only Delly, and both tools), while fluorescence *in situ* hybridization (FISH) results were shown as green for positive cases and gray for negative. Cases without FISH data are not included. (C) Pearson correlation test among the variant allele frequency (VAF) of the variants identified in the replicates. DNA were colored based on the reason for discordance with the replicate after manual review. (D) Pie charts showing the genes' variant frequencies in each cohort. The internal circle represents the frequency of variants in the BO cohort, while the external circle represents the variants in the MI cohort. (E) Pie chart showing the total variants concordance between cohorts. Variants called in both BO and MI samples in blue; variants called only in the BO samples in red or only in the MI samples in orange. CNA: copy number alteration; Amp C: concordant amplification; Amp D: discordant amplification; Del C: concordant deletion; Del D: discordant deletion.

plementing an additional variant calling tool able to reliably call SNV in the absence of germline controls. Additionally, the risk of losing sub-clonal variants can be avoided by increasing the sequencing coverage.

MI-BO analysis pipeline comparison

The MI-BO cohort of patients included 20 samples from 20 patients that had been previously analyzed by different approaches in Milan (see *Online Supplementary Methods*) and whose results were compared with those obtained from the same samples by employing the UMA panel. In detail, the SNV calls were compared between the two targeted NGS strategies used, while UMA t-IgH and CNA calls were compared with the Milan FISH and ULP-WGS results, respectively.

The CNA profiles detected by the UMA panel resulted overall concordant with those detected by ULP-WGS (mean concordance: 89.23%) (Figure 5A). The least concordant profiles were those of samples ID3867 and ID3859 (concordance: 80-90%) and were caused by sub-clonal broad amplifications called by the UMA-panel analysis pipeline, whereas they were undetected for the ULP-WGS analysis pipeline. FISH data for t-IgH was available for 16/20 samples. A 99% agreement between the two t-IgH calling strategies was obtained, with no t(4;14) or t(14;16) detected. The only discordance was highlighted for a t(11;14), called positive by FISH and negative by the UMA panel (Figure 5B).

The comparison of SNV was limited to genes present in both panels used in Milan and in Bologna. Overall, a good concordance between the two panels was achieved (R=0.58, *P*=0.0039) (Figure 5C), with 18/24 (75%) somatic variants detected by both sequencing approaches. Discordances (6/24, 25% SNV detected from the MI panel only: Only MI panel) were related to Mutect filters and to regions of poor read coverage, as previously described (Figure 5D). Importantly, all known MM pathogenic variants (N=8) were correctly detected by both approaches (Figure 5E).

Identification of high-risk patients by the Unique Molecular Assay panel

Finally, the genomic information gathered by the UMA panel was exploited to define the patients' prognostic risk, according to the most recently published staging systems, as well as to their updated revisions (i.e., ISS, R-ISS, R2-ISS, respectively), which combine both clinical and genomic information to stratify patients in risk classes.[3,4] Notably, the critical role of several genomic risk factors (i.e., *TP53* mutated, del(17p), gain(1q), del(13q), del(1p), t(4;14), t(14;16), and t(14;20)) in determining MM patients' risk and prognosis has been also confirmed in several independent studies.[9-13,37] To this purpose, according to the UMA panel results, we assigned a R2-ISS risk class to patients included in the study, provided that the clinical parameters required to calculate the risk were available (i.e., to 98/130 patients from the BO cohort). In addition, we described the previously mentioned genomic risk factors harbored by patients within each class. Overall, 98 genomic risk factors were detected; this number tended to increase throughout the R2-ISS risk classes (Figure 6A). A co-occurrence analysis of the risk factors in single patients (Figure 6B) revealed that in the R2-ISS "High" group, most patients carried ≥2 genomic risk factors (16 out of 20), with the association between gain(1q) (*CKS1B*) and del(13q) (*RB1*) being the most frequently observed co-occurrence (11 out of 16 patients), as recently reported in high-risk patients.[13,38] Notably, the NGS analysis allowed us to detect additional risk factors also in the R2-ISS lower risk classes, such as two del(1p) (*CDKN2C*) events in the R2-ISS "Low" class, and one double-hit *TP53* event (mutation + deletion) in the R2-ISS "Low-intermediate" class. These cases might represent a

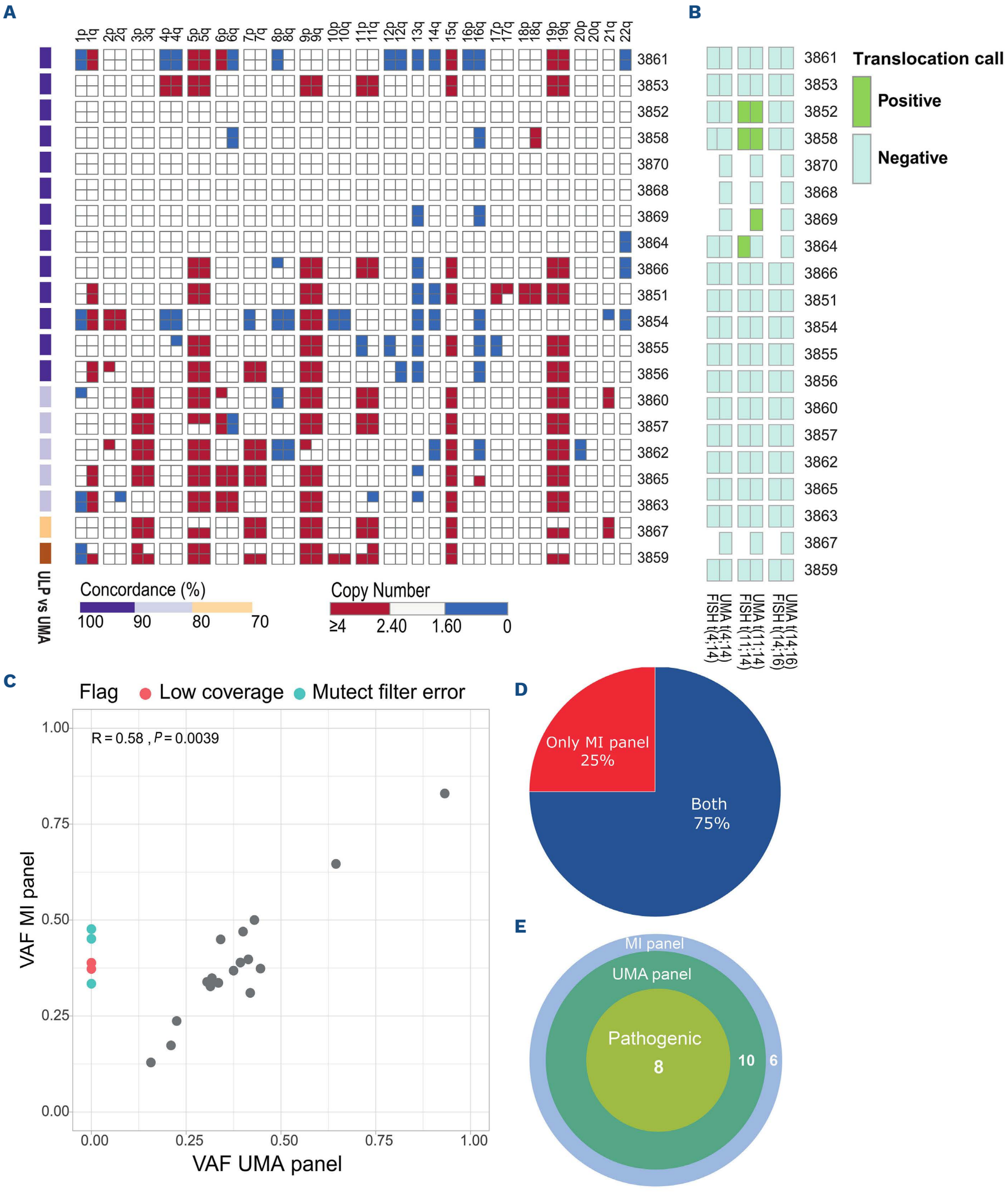

Figure 5. Bologna-Milano interlab validation results. (A) The plot displays the copy number (CN) profiles of the Bologna-Milano (BO-MI) cohort DNA, overlaying the BO sample (sequenced with the the Unique Molecular Assay [UMA] panel, top line) on the MI replicate (sequenced with ultra-low pass whole genome sequencing [ULP-WGS], bottom line). Amplifications above 40% clonal-

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ity are shown in red, while deletions below 40% are in blue. (Left) Concordance rate of the pairwise matching analysis at the segment level (ULP-UMA CN matching). Concordance percentages are displayed on a scale from purple to yellow. (B) Translocations (t) involving the IGH-region (t-IgH) were evaluated: t(4;14), t(11;14), t(14;16), and compared with MI fluorescence *in situ* hybridization (FISH). Cases without MI FISH data are not included. (C) Pearson correlation test among the variant allele frequency (VAF) of the variants identified in the replicates. Samples were colored based on the reason for discordance with the replicate after manual review. (D) Pie chart showing the total variants concordance between the UMA panel and MI panel; variants called in both the UMA panel and MI panel samples in blue, variants called only in the MI samples in red. (E) Pie chart showing that 18 variants were detected by both panels and, of these, 8 are pathogenic (affecting *TP53* and *NRAS*); the group of single-nucleotide variants (SNV) found only by the MI panel are shown in light blue, those shared with the UMA panel in green, and pathogenic variants in light green. CNA: copy number alteration.

small subset of high-risk patients that could be misclassified by the R2-ISS staging system.
Taken together, these results suggest that, by using the UMA panel, it might be possible to correctly define the MM risk classes according to the most updated scoring systems, thus supporting reliable patient stratification.

Discussion

This study presents the validation and the implementation of the UMA NGS panel, designed for comprehensive and resource-efficient genomic characterization and risk stratification of MM patients. Our findings demonstrate that the UMA panel provides detailed genomic profiling by accurately detecting canonical t-IgH, mutations in MM-critical genes, as well as gene-level and whole-genome CNA.
The design of the UMA panel represents an accessible, agile, and cost-effective solution that provides comprehensive genomic information at limited costs, in contrast to previous approaches. In fact, the cost-benefit ratio of the UMA panel is achieved through two key factors: 1) the sequencing procedures, which take advantage of a small panel design to efficiently profile patient samples, thus reducing both sequencing costs and reporting times of results compared to other methods; and 2) the bioinformatic pipeline, which utilizes all sequencing reads (both off-target and on-target) to accurately identify whole-genome and gene-level CNA. Additionally, the targeted design of the informed t-IgH regions effectively captures all known IgH translocation hotspots, ensuring robust detection of translocations while minimizing the panel footprint. By integrating these strategies into a single assay, the UMA panel delivers a comprehensive genomic overview while maintaining cost-effectiveness. This makes it particularly well-suited for routine clinical use, where balancing budget constraints with the need for thorough genomic information is crucial.
A pivotal aspect of this study was the validation of the UMA panel's NGS results through direct comparison with data obtained from the same samples using FISH, the gold-standard technique for patient characterization in MM. This comparison ensured the accuracy and reliability of the UMA panel in clinical settings. The comparison showed that the UMA panel performances were on a par with the reference method in detecting alterations, with an UMA panel accuracy of over 93% in both CNA and t-IgH calling. This underscores the UMA panel's reliability and suggests its potential to replace traditional methods in clinical settings requiring detailed genomic profiling. Notably, the few detected discordances observed between the UMA panel and FISH results could be attributed to inherent methodological differences and the specific design of our experimental setup. Furthermore, despite the fact that FISH was used as gold-standard here, FISH itself is far from offering 100% accuracy. Failures due to limitations in cell number, cell purity or technical reasons are, indeed, common occurrences in all FISH laboratories.
During the inter-laboratory validation of the UMA panel, the BO-MI analysis results emphasized the panel's robustness and consistency across different laboratory settings. The cross-validation confirmed also that the UMA panel maintains optimal performance when implemented in different environments, demonstrating excellent concordance in genomic alteration detection across sites. However, the study also revealed challenges, such as minor variations in sequencing output and metrics due to differing local practices, which required adjustments to the bioinformatic pipelines for consistent data interpretation. But the MI-BO inter-laboratory analysis demonstrated that data from the UMA panel is highly concordant with results from alternative approaches, utilizing a different sequencing and bioinformatic analysis.
Taken together, these findings crucially affirm the robustness of the UMA panel's approach and its utility in broader clinical settings. The UMA can be reliably used across different centers without significant loss of data reliability or diagnostic accuracy. This validation phase provided valuable insights into the panel's scalability and reinforced its potential for widespread clinical deployment. Future research should focus on expanding the UMA panel's validation across additional treatment centers to further establish its broad applicability and robustness. Additionally, it would be worthwhile to explore the potential of including genes encoding antigens targeted by emerging immunotherapies, such as chimeric antigen receptor T cells and bispecific antibodies, within the panel. This could further expand its utility in the evolving landscape of personalized medicine.

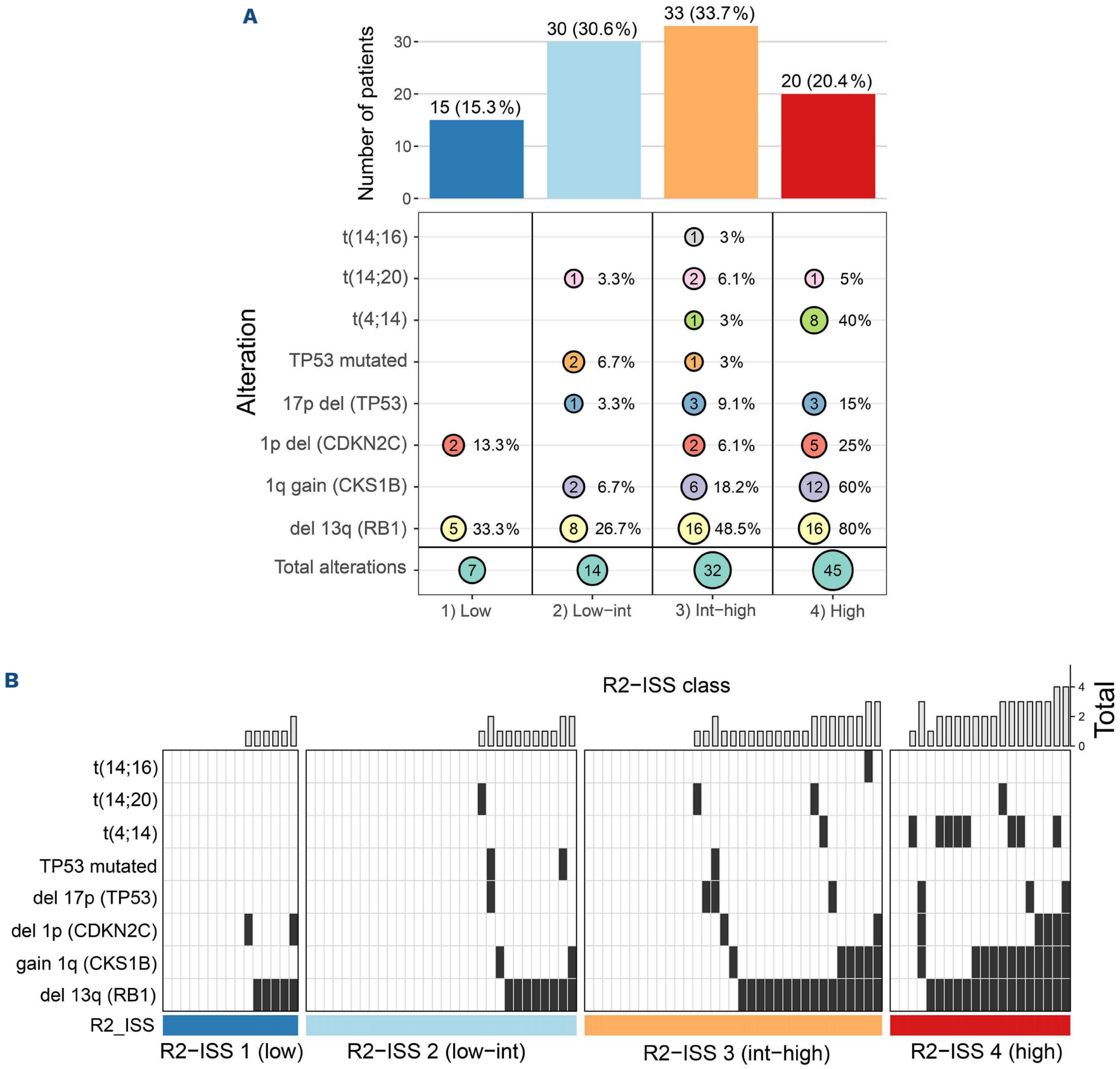

Figure 6. Unique Molecular Assay panel usage for multiple myeloma risk stratification. (A) Dotplot summarizing the main genomic risk factors identified by the Unique Molecular Assay (UMA) panel in the Bologna (BO) cohort, broken down by Second Revision of the International Staging System (R2-ISS) classes (only patients [pts] with available R2-ISS are showed, N=98). A histogram describing the classes' frequencies is to be found above the dotplot. (B) Risk factors-patients matrix which illustrates the co-occurrence of risk factors within single patients. It is possible to observe the overall increase of detected risk-factors in higher risk classes. MM: multiple myeloma; del: deletion; Int: Intermediate, t: translocation.

The extended genotyping provided by the UMA may well have a scope beyond NDMM. Indeed, risk stratification in smoldering multiple myeloma (SMM) has gained significant interest thanks to a comprehensive genomic profiling of this entity.[39-42] Current risk stratification relies on surrogates of disease burden coupled with few FISH abnormalities,[43] yet it is increasingly clear that NGS analysis my further improve stratification of patients with implications for treatment decisions.[44] However, the main strength of our paper relies in validating the UMA panel in identifying high-risk patients with MM. In fact, by utilizing the UMA, we were able to stratify patients effectively into risk categories outlined by the R2-ISS. Additionally, this genomic profiling facilitated the detection of additional risk factors, such as *TP53* mutations/deletions and co-occurrence of chromosomal deletions and gains[9,13,38] that are crucial for determining prognosis and guiding treatment strategies. The UMA can, therefore, go beyond current diagnostics

and easily adapt to future revisions of risk stratification or identification of novel predictive markers. This can significantly impact clinical decision-making, allowing for more personalized, and potentially more effective, treatment approaches. Moreover, the UMA panel's ability to identify additional risk factors in lower-risk groups demonstrates its potential to redefine risk stratification paradigms and influence early intervention strategies.

In conclusion, the UMA panel represents a significant advancement in the molecular diagnostics of MM, offering a robust and cross-validated tool for enhancing personalized medicine strategies. Its implementation could lead to a shift towards more genetically informed decision-making, underscoring the critical role of continuous technological innovation in the fight against MM. As we refine and further validate this tool, it holds the promise to improve patient outcomes through tailored interventions and to serve as a model for future advancements in MM molecular diagnostics.

Disclosures

MC has received honoraria from Amgen, AbbVie, Bristol-Myers Squibb, Celgene, GlaxoSmithKline, Janssen, Menarini-Stemline, Pfizer, and Sanofi. EZ has received honoraria from Janssen, Bristol-Myers Squibb, Sanofi, Amgen, GlaxoSmithKline, Pfizer, Oncopeptides, and Menarini-Stemline. NB has received honoraria from Janssen, Pfizer, GlaxoSmithKline, Jazz, and Takeda. All the other authors have no conflicts of interest to disclose.

Contributions

AP conceptualized and designed the study, analyzed data, performed bioinformatics analyses, and wrote the manuscript. BT designed the study, analyzed data, processed samples, and wrote the manuscript. GMaz and VMV analyzed data, performed bioinformatics analyses, and wrote the manuscript. ML and MM designed the study, analyzed data, processed samples, and critically revised the manuscript. GMar, AMae and SF processed the samples and analyzed data. VS, IV, EB, SA and IP analyzed data and critically revised the manuscript. AMar analyzed data and performed bioinformatics analyses. PT, KM, IR and LP collected the samples and reviewed clinical data. NT analyzed data and reviewed clinical data. MC and EZ critically revised and approved the manuscript. NB and CT conceptualized and designed the study, analyzed data, and critically revised and approved the manuscript.

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Data-sharing statement

The datasets and analysis code generated during the current study are available from the authors on reasonable request in a dedicated GitHub repository: https://github.com/andrea-poletti-unibo/paper_UMA_panel.

The raw DNA sequencing data generated during the current study are not publicly available due to patients not authorizing the public sharing of data.

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