Multiple myeloma (MM) is a plasma cell malignancy that is heterogeneous in nature and comprises various molecularly distinct subtypes (1). Despite the advances in therapies, drug resistance and multiple relapses are the disease’s hallmarks, contributing to early deaths. Combining therapies and identifying prognostic biomarkers are indispensable for enhancing the outcome of relapsed and/or refractory MM. To achieve this, the incorporation of sophisticated techniques to discover molecular biomarkers in clinical trials is a must. Such biomarkers can lead to better understanding of the molecular mechanisms contributing to drug resistance and the identification of targets for therapies that avert these resistance mechanisms. Next-generation sequencing and single-cell RNA sequencing (scRNASeq) are being explored as tools in assessing the prognosis of MM and have proven fruitful in dissecting complex molecular dynamics of biological phenomena like resistance and relapse.

In a recent multicenter clinical trial, scRNASeq was performed to extensively investigate MM resistance to a 4 drug combination regimen: daratumumab, carfilzomib, lenalidomide, and dexamethasone (DCLD) (2). A cohort of 41 patients, either with primary resistance to bortezomib (RRB) proved to be effective. The overall response rate was 88%, and the median progression-free survival (PFS) was 14.7 months. The overall survival was not reached during the median follow-up time of the trial (14.6 months), while median overall survival of MM resistance to bortezomib (RRB) proved to be effective. Additionally, metacell clustering of PCs in each patient vs the same number of PCs from the HD cohort was performed to define transcriptionally homogeneous clones, and their clonality was validated by the presence of single VDJ arrangements; the method is shown graphically in Fig. 1. Transcriptomic expression of MM driver genes, including the common ones; TXNIP, MIZB1, CD38, CCND1, CCND2, SDC1, and TNFRSF17, did not show a significant difference ($P > 0.05$) between NDMM and RRB, nor was a difference in clonality observed, suggesting that resistance mechanism exists beyond, the commonly known MM driver gene set and hence they cannot be used to predict drug resistance or relapse.

In their quest for discovering a resistance mechanism, the authors used a bootstrapping strategy to quantify the expression between MM cells in a specific clone vs HD. A total of 66 genes were found to be differentially expressed and clustered into 3 modules distinguishing RRB from NDMM:

1-Module 1 consisted of 24 genes containing previously described high-risk genes (STMN1, PSMB4, RR2, and TYMS) and new genes such as PPIA. At the functional level, module 1 was enriched in mitochondrial, endoplasmic reticulum (ER), and unfolded protein response pathways. Genes in Module 1 were expressed less in NDMM compared to RRB. The RRB cohort was subdivided into groups 1 and 2, based on the expression gradient of module 1 genes being low in group 1 and high in group 2, respectively.

2-Module 2 consisted of 17 genes mainly enriched in the tricarboxylic acid cycle and oxidative phosphorylation (OXPHOS) pathways.

3-Module 3 consisted of 25 genes. Interestingly, RRB patients with overexpressing module 1 (group 2) signature were downregulating module 3.

The strategy of integrating scRNASeq in the clinical trial was proven successful in clearly stratifying group 2 of RRB (module 1 overexpression) from NDMM and group 1 of RBB. A future clinical approach could involve using the module 1 signature to predict resistance to DCLD combination.
The authors explored whether RRB resistant signature can predict DCLD regime outcome. They first used PFS analysis, to determine whether cytogenetic risk factors (double hit vs C21 double hit) could predict DCLD outcome in RRB. Second, the same analyses were performed in module 1 low vs high groups. The median PFS for C21 double hit was 4.9 months, and for module 1 high, it was 4.3 months. Thus, patient stratification based on the RRB resistant signature outperformed the cytogenetic risk stratification. As expected, integration of both double hit and module 1 high in multivariate analyses increased the prediction power of calculating outcomes of the DCLD treated patient. Additionally, in an independent dataset of 1150 MM patients from 76 sites worldwide called the CoMMpass dataset, module 1 high was predictive for poor response and early progression on DCLD (<18 months) in 72% of cases. Finally, module 1 high patients in CoMMpass had several clinical and biological risk factors associated with them. These results show that the module 1 gene signature is an effective predictor of DCLD outcome and a biomarker of drug resistance that potentially can be used in the clinic.

The RRB cohort was further subdivided into non-responders (<partial response at 6 months) and responders to DCLD treatment, and differentially expressed analysis revealed 133 genes clustered in 2 modules. These modules were enriched for putative MM resistance pathways, specifically related to increased nucleoside metabolism and decreased protein processing in the ER, indicating possible escape mechanisms of these cells from DCLD treatment. This signature significantly overlaps with the tumor resistance signature of RRB (P = 3.5 × 10⁻⁷), and nonresponders significantly overlapped to the module 1 signature of RRB (P = 0.029). A neural network-based regression analysis predicted patients responding (n = 24) to treatment after 4 months, meaning that detected resistant mechanisms are shared and generalized to larger cohorts.

The authors then performed longitudinal scRNAseq analysis (at baseline and treatment cycles 4 and 10) on patients who responded to DCLD but later relapsed, and then combined these results with clonal analysis using B-cell receptor sequencing of each clone. There were 3 trajectories; first, in patients responding to treatment for most malignant PCs replaced with healthy PCs; second, nonresponders retained >50% of malignant PCs. The third trajectory was patients with clonal selection. Results reflect what was expected and explanatory to clinical findings. Confirmatory to literature, CD38 was consistently downregulated in the resistant clones, and myeloid cell program genes S100A10 and S100A11 were upregulated. Interestingly, machine
learning analysis revealed that the acquisition of drug resistance in MM might occur in multiple stages or on smaller clones.

Finally, the authors selected *PPIA*—a gene involved in efficient protein folding—that was upregulated in resistant patients for further characterization. It was hypothesized that *PPIA* might induce drug resistance by accelerating the protein folding pathway, thus reducing tumor toxicity induced by proteasome inhibitors. Knockout of *PPIA* or its chemical inhibition in a MM malignant cell line rendered it nonresistant to carfilzomib compared to wild type. Thus, inhibitors of *PPIA* combined with proteasome inhibitors might be a promising therapeutic strategy to overcome drug resistance in MM. The combination of carfilzomib and cyclosporin or cyclosporine A was effective in induction of apoptosis in a MM cell line and induced genes involved in ER stress response and apoptosis signaling pathways while downregulating housekeeping and metabolic genes. The same combination was also found to be synergistic in inducing apoptosis in PCs, ex vivo, of a resistant patient from the RRB cohort.

Clinical use of these newly discovered signatures, specifically module 1 high of patients who were RRB, helps predict a group of patients at risk of drug resistance. This method could be implemented using techniques such as qPCR and also by machine learning-based methods, as suggested. However, group 1 (module 1 low) of the patients who were RRB was not clearly distinguished from NDMM; rationally, a more powerful method to segregate these patients better from NDMM is needed. The cost of scRNASeq is another hurdle in predicting drug resistance from the RRB cohort.

In short, this article elegantly reiterates that clinical trials using scRNASeq can unleash a wealth of minute details related to dynamic molecular changes explaining tumor resistance and identifying potential therapeutic targets. The resistant signatures and methods provided by this study successfully predicted the group of patients who were resistant to the DCLD combination and even outperformed the routinely used immunohistochemistry-based stratification.

**Nonstandard Abbreviations:** MM, multiple myeloma; scRNASeq, single-cell RNA sequencing; DCLD, daratumumab, carfilzomib, lenalidomide and dexamethasone; RBB, relapsed or refractory to bortezomib; PFS, progression-free survival; PCs, plasma cells; NDMM, newly diagnosed MM; HD, healthy donors; ER, endoplasmic reticulum.

**Human Genes:** TXNIP, thioredoxin interacting protein; *MZB1*, marginal zone B and B1 cell-specific protein; *CD38*, *CD38* molecule; *CCND1*, cyclin D1; *CCND2*, cyclin D2; *SDC1*, syndecan 1; *TNFRSF17*, TNF receptor superfamily member 17; *STMN1*, stathmin 1; *PSMB4*, proteasome 20S subunit beta 4; *RRM2*, ribonucleotide reductase regulatory subunit M2; *TYMS*, thymidylate synthase; *PPIA*, peptidylprolyl isomerase A; *S100A10*, S100 calcium binding protein A10; *S100A11*, S100 calcium binding protein A11.

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